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THE EFFECT OF ADRENALECTOMY ON THE SUCCINIC DEHYDROGENASE ACTIVITY AS RELATED TO NITROGEN AND OTHER COMPONENTS OF RAT LIVER TISSUE [1]

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INTRODUCTION

As early as 1913 (Gradinescu, '13) the fact was established that the adrenal cortex plays a role in the maintenance of the normal metabolic rate. Other investigators were able to substantiate this fact in a variety of animals (Marine and Baumann, '22; Aub et al., '22; Artundo, '27a). Estrada ('27) found that in the adrenalectomized dog the oxidative processes of the muscles were significantly lowered. The in vitro oxygen consumption of kidney and liver slices was also markedly reduced in adrenalectomized animals (Crismon and Field, '40; Russell and Wilhelmi, '41; Tipton, '41). Tipton found, however, that addition of substrate to the medium containing the slices raised the Q_{O_2} to a normal level and he suggested that the substrate may have been the limiting factor. Himwich, and co-workers ('34), and Sass-Kortsak ('44) studied the metabolism of brain tissues from adrenalectomized animals and concluded that the change in substrate conditions rather than in the capacity of the cell resulted in the lowered rate of oxygen uptake.

The enzymes, as limiting factors in the cell, may also be markedly altered in adrenalectomy. Tipton ('44) found that adrenalectomy in rats resulted in a decreased Q_{O_2} in a system designed to make cytochrome oxidase the limiting factor,

[1] This investigation was supported in part by a research grant from the National Institutes of Health and the Wisconsin Alumni Research Foundation.

when tested in kidney, heart and liver tissues. He also found a reduction in the quantity of cytochrome c in these same tissues. Tipton, Leath, Tipton and Nixon ('46) later obtained a reduction in Q_{O_2} when measuring the succinoxidase system in the livers of adrenalectomized rats. The rate of oxidation of pyruvate, succinate and citrate by the kidney is also reduced after adrenalectomy (Russell and Wilhelmi, '41).

The study presented here was undertaken to examine some of the relationships between apparent enzyme activity changes and changes in tissue composition occurring after adrenalectomy. We chose to investigate succinic dehydrogenase (SDH-ase) activity in liver tissue of the albino rat and to relate it to the nitrogen, lipid and water content of the tissue since it has been shown that in at least one strain of rats, adrenalectomy results in an apparent decrease in the liver SDH-ase concentration (Tipton, Leath, Tipton and Nixon, '46).

METHODS AND MATERIALS

The animals used in the following experiments were albino rats of the Holtzman-Rolfsmeyer strain. The animals were used within two weeks after receipt from the breeder, and were fed Rockland Rat Diet, ad libitum. In all of the experiments, both unoperated controls and adrenalectomized rats were used.

For the SDH-ase determinations, liver tissue was prepared by homogenization in glass distilled water. A 2.5% homogenate was used. The activity of the enzyme in a sample of the homogenate was measured using the conventional Warburg respirometer and was expressed in cubic millimeters oxygen uptake per 10 minutes per 0.4 ml of a 2.5% homogenate (h), in cubic millimeters of oxygen taken up per hour by the quantity of fresh tissue equivalent to 1 mg of dried tissue (Q_{O_2}) or the hourly oxygen uptake of a quantity of tissue equivalent to 1 mg of tissue nitrogen ($Q_{O_2}[N]$).

The concentrations of the reagents in the fortified reaction medium were such that SDH-ase was the limiting factor in the reaction (Schneider and Potter, '43). It has been shown

that in normal and adrenalectomized animals the quantity of cytochrome oxidase in the tissue is more than sufficient to oxidize all the cytochrome c reduced in the dehydrogenation of the succinic acid (Schneider and Potter, '43; Tipton et al., '46). Manometer readings were taken every 10 minutes for 40 minutes.

Another portion of the tissue homogenate was transferred to a tared vial and dried in an oven at 72°C. for a determination of the dried weight of the tissue. In some cases, homogenate samples were taken for nitrogen analysis and in one experiment tissue homogenates were analyzed for both total nitrogen and non-protein nitrogen. The non-protein nitrogen fraction of the homogenate was represented by nitrogen compounds not precipitated by TCA (trichloracetic acid). Nitrogen was determined by a modification of the method of Johnson ('41). When fat determinations were made on liver samples, the procedure was that presented by Payne ('49).

RESULTS AND DISCUSSION

The initial experiment was designed to determine whether or not adrenalectomy produced a change in the concentration of liver SDH-ase in animals of the Holtzman-Rolfsmeyer strain. The procedure used in this experiment was as follows: 90 day, female, albino rats were divided into three groups. Group I served as controls while the animals in groups II and III were bilaterally adrenalectomized and kept for periods of 8 and 16 days, respectively, before enzyme determinations were made. Animals in the experimental groups received no special post-operative care.

Our data indicate a rise in oxygen uptake (table 1). Tipton, Leath, Tipton and Nixon ('46) found that adrenalectomy in the rat resulted in a lowered oxygen uptake when the activity of SDH-ase was measured in liver tissue. Koepf and co-workers ('41) found that the oxygen uptake of liver slices was increased after adrenalectomy and more recently Wollman and Scow ('51) demonstrated that adrenalectomy re-

sulted in a slight but not significant rise in Q_{O_2} when SDH-ase was measured in liver tissue. Increases in oxygen uptake of 18 and 38% were observed in our adrenalectomized animals. Application of Fisher's t test shows a significant difference between the values obtained for the control group and group II and a highly significant difference between the control group and group III. It can be concluded that on a dry weight basis there was an increase in SDH-ase concentration in livers of the adrenalectomized animals. There is, however, an immediate objection to the assertion that this means increased enzyme synthesis. It is possible that the level of

TABLE 1

Liver SDH-ase activity in mature female rats

	GROUP	Q_{O_2}	COMPARISON BETWEEN	P [1]	% CHANGE
I	Controls (5) [2]	76.9 ± 2.8 [3]	.....	..	...
II	Adrenalectomized 8 days (4)	90.7 ± 5.4	I, II	.05	18.0
III	Adrenalectomized 16 days (5)	106.2 ± 8.0	I, III	.01	38.0

[1] Probability that difference between indicated groups is due to chance.
[2] Figures in parentheses are numbers of rats.
[3] Mean ± standard error of the mean.

enzyme and its rate of synthesis may remain normal or even drop below normal in states of insufficiency, but that this is not reflected in an estimation of activity based on tissue dry weight. The next experiment was designed to consider this problem.

It is suggested that a measurement of SDH-ase activity on the basis of total nitrogen might give a better indication of increased or decreased enzyme synthesis in adrenal insufficiency. The assumption is made that if increased synthesis occurs in the oxidative enzymes, the increase will not be paralleled by increased synthesis of all other protein constituents of the tissue. Two groups of animals were used

in this experiment. Group I animals were kept as controls and group II animals were bilaterally adrenalectomized 12 days before sacrifice. Enzyme activity measurements were made as before. Tissue dry weights and total nitrogen values were determined. The results are given in table 2.

TABLE 2

SDH-ase activity and nitrogen concentration in livers of normal and adrenalectomized mature female rats

MEASUREMENT	GROUP I CONTROLS (2)[1]	GROUP II ADRENALECTOMIZED 12 DAYS (4)	P[2]	% CHANGE
h value	37.6 ± 2.3[3]	41.0 ± 1.7	.25	9.0
Dry weight per ml 2.5% homogenate (mg)	7.55 ± 0.25	6.78 ± 0.13	.05	10.4
Q_{O_2}	74.7 ± 2.1	90.9 ± 2.9	.025	21.6
Nitrogen per ml 2.5% homogenate (mg)	0.695 ± 0.015	0.711 ± 0.031	.8	2.3
Nitrogen per mg dry weight (mg)	0.0922 ± 0.0010	0.1046 ± 0.0034	.08	13.3
Q_{O_2} (N)	812 ± 31	870 ± 9	.07	7.0
% protein concentration in solids (total nitrogen per mg dry wt. × 6.25 × 100)	57.6 ± 0.6	65.4 ± 2.1	.08	13.3

[1] Figures in parentheses are number of rats.

[2] Probability that difference between control and adrenalectomized groups is due to chance.

[3] Mean ± standard error of the mean.

Examination of the data (table 2) indicates that the enzymic activities of equivalent quantities of fresh tissues (h value) taken from normal and adrenalectomized animals were approximately the same. The water content of the tissue from the adrenalectomized animals was increased to a significant extent, hence the Q_{O_2} was also increased. This find-

ing is consistent with an earlier observation of hydration in the liver and skin of the rat after adrenalectomy (Hartman, '33). Owing to the small number of animals used in this experiment, the relationship between oxygen uptake and tissue nitrogen is not well defined. The data indicate increases in tissue nitrogen both on a dry weight and wet weight basis but these increases are not statistically significant. The increase is responsible for the similarity between the $Q_{O_2}(N)$ values for the control and adrenalectomized groups.

In the experiments discussed thus far, we used mature female rats as experimental animals. Other investigators (Tipton, Leath, Tipton and Nixon, '46) studying SDH-ase in livers of adrenalectomized rats used immature males and provided these animals with 1% NaCl for three days after the operation. It was suggested that with our strain of rats these modifications might produce results differing from those we obtained. Therefore, the procedure in the following experiment was as follows: Male rats weighing from 70 to 100 gm were divided into control and experimental groups. The control animals were left intact and were given tap water to drink. After adrenalectomy the experimental animals were given 1% NaCl to drink for the first three post-operative days. After that time they received ordinary tap water. On the 6th and 7th day following adrenalectomy the animals were sacrificed and a portion of the liver removed and homogenized. Enzyme determinations were made on portions of the fresh homogenates and portions were prepared for total nitrogen analyses as in the second experiment. The results are presented in table 3.

These data reveal a highly significant increase in the Q_{O_2} values of the adrenalectomized as compared to the control animals. This change is comparable to that of the adrenalectomized mature female rats in the previous experiment. Tissue hydration has occurred to a significant extent. Again there has been an increase in the concentration of nitrogen in the tissue of the adrenalectomized animals, and the differences are highly significant both when the nitrogen

is expressed in terms of milligrams of dry weight of tissue, or in terms of milliliters of fresh 2.5% homogenate, i.e., wet weight of tissue. The increase in nitrogen per milliliter of homogenate was 12.8%. A corresponding significant increase of 11.4% in the average h value can be seen. This could be

TABLE 3

SDH-ase activity and nitrogen concentration in livers of normal and adrenalectomized immature male rats

MEASUREMENT	GROUP I CONTROLS (6)[1]	GROUP II ADRENALECTOMIZED 6–7 DAYS (10)	P[2]	% CHANGE
h value	40.3 ± 1.4[3]	44.9 ± 1.3	.05	11.4
Dry weight per ml 2.5% homogenate (mg)	7.18 ± 0.20	6.47 ± 0.19	.04	9.8
Q_{O_2}	85.12 ± 2.99	104.93 ± 4.30	.01	23.2
Nitrogen per ml 2.5% homogenate (mg)	0.7448 ± 0.0060	0.8404 ± 0.0053	.0001	12.8
Nitrogen per mg dry weight (mg)	0.1042 ± 0.0073	0.1308 ± 0.0041	.0005	25.5
Q_{O_2} (N)	818 ± 33	802 ± 24	.75	1.9
% protein concentration in solids (total nitrogen per mg dry wt. × 6.25 × 100)	65.1 ± 1.4	81.8 ± 2.5	.0005	25.5

[1] Figures in parentheses are number of rats.

[2] Probability that difference between control and adrenalectomized groups is due to chance.

[3] Mean ± standard error of the mean.

expected if the rate of enzyme synthesis remained unchanged relative to the rate of synthesis of other protein constituents of this tissue. Thus, if the oxygen uptake per unit of homogenate were to increase, we might expect a corresponding increase in the amount of protein per unit of homogenate.

The parallel increases in O_2 uptake and tissue nitrogen support such a conclusion.

The same analysis can be applied to oxygen uptake per unit of tissue solids. Q_{O_2} is an expression of oxygen uptake per milligram dry weight and its increase under the conditions of adrenal insufficiency amounted to 23.2%. A corresponding increase of 25.5% is seen in the amount of nitrogen per dry weight of tissue. Thus when oxygen uptake is expressed in terms of cubic millimeters oxygen per milligram nitrogen per hour, $Q_{O_2}(N)$, there is no difference between the controls and the adrenalectomized animals.

It would appear from the data that with our strain of rats, sex and age differences do not essentially alter the relationships between liver SDH-ase activity in normal and adrenalectomized animals. In both immature males and sexually mature females, adrenal insufficiency does not cause a change in the activity of SDH-ase when such activity is measured in terms of tissue nitrogen.

There remains at least one other important consideration. If the concentration of protein in the tissue solids is seen to increase under conditions of adrenal insufficiency, there must be a corresponding decrease in the relative concentration of one or more of the other tissue constituents. Early investigation (Schwarz, '10), well substantiated by more recent work (Artundo, '27b; Cori and Cori, '28), have shown that adrenalectomy in the rat results in almost complete depletion of liver glycogen. Silvette ('34) observed a progressive fall in the chloride content of the liver of the cat after adrenalectomy and Marenzi ('38) found decreased potassium in the liver of the rat after adrenalectomy. The decreases in carbohydrate and inorganic salt levels may or may not entirely account for the relative increase in protein. It is possible that the lipid concentration in the tissue may change as well. Furthermore, it may be an erroneous assumption that all of the increased tissue nitrogen represents protein. It is certain that at least part of the nitrogen found in the tissue homogenates comes from non-protein tissue and blood

constituents, and it is possible that the concentration of these non-protein constituents change in adrenal insufficiency quite independently of the changes in protein concentration. If the increase in total nitrogen in the tissue of an adrenalectomized animal represented an increase in non-protein constituents, our data would support the hypothesis that an actual increase in rate of enzyme synthesis occurred relative to the rate of synthesis of other proteins. The next experiment was designed to consider the changes in lipid and non-protein nitrogen concentrations.

The control and experimental animals again were immature males ranging in weight from 70 to 100 gm. The procedure followed was the same as in the previous experiment until the time of sacrifice.

When the animals were sacrificed, a sample of liver was taken from each and immediately frozen in a refrigerator. This sample was preserved for a fat analysis. Another liver sample was homogenized with sufficient cold distilled water to make a 5% homogenate. One milliliter of the homogenate was diluted with 19 ml of water and a 0.5 ml aliquot of this mixture was analyzed for total nitrogen. Five milliliters of the 5% homogenate was then placed in a small beaker and 5 ml of a 10% TCA solution added with agitation. The mixture was centrifuged and the supernatant decanted. A 0.5 ml aliquot of the supernatant was analyzed for nitrogen. The difference between the total nitrogen and the nitrogen found in the supernatant will be assumed to represent total protein nitrogen though this value includes a few other nitrogenous compounds such as the pentose nucleic acids and some non-precipitating nitrogenous substances which are carried down with the precipitate and the cellular debris. The results of the experiment are presented in table 4.

The non-protein nitrogen concentration in the livers from the experimental animals shows a highly significant increase of 34.3% over the control value. Part of the increase is possibly due to accumulation of non-protein nitrogenous compounds in the blood and probably part is due to the ac-

TABLE 4

Nitrogen and lipid liver constituents of normal and adrenalectomized immature male rats

MEASUREMENT	GROUP I NORMAL CONTROLS (8)[1]	GROUP II ADRENALECTOMIZED 6–7 DAYS (15)	P[2]	% CHANGE
Dry weight per ml 5% homogenate (mg)	15.08 ± 0.18[3]	14.23 ± 0.11	.0003	5.7
Total nitrogen per ml 5% homogenate (mg)	1.569 ± 0.051	1.771 ± 0.030	.002	12.9
Non-protein-nitrogen per ml 5% homogenate (gamma)	98.2 ± 2.1	131.9 ± 6.1	.001	34.3
Protein nitrogen per ml 5% homogenate (mg)	1.471 ± 0.049	1.639 ± 0.028	.005	11.4
Protein nitrogen per mg dry weight (mg)	0.0977 ± 0.0039	0.1151 ± 0.0017	.0001	17.8
% protein concentration in solids (protein nitrogen per mg dry wt. × 6.25 × 100)	61.0 ± 2.4	71.9 ± 1.1	.0001	17.8
% lipid concentration in wet weight	3.97 ± 0.41	4.23 ± 0.18	.5	6.5
Per cent of wet weight represented by solids:			Group I 30.2% Group II 28.4%	
Per cent of solids represented by lipid:			Group I 13.1% Group II 14.9%	
Per cent of solids represented by protein and lipids:			Group I 74.1% Group II 86.8%	

[1] Figures in parentheses are number of rats.

[2] Probability that difference between control and adrenalectomized groups is due to chance.

[3] Mean ± standard error of the mean.

cumulation of similar compounds in the cells and/or in the interstitial fluid. In this experiment the level of protein nitrogen rose 11.4% when the measurement was based on wet weight of tissue, i.e., protein per milliliter of homogenate. In our third experiment an increase of 11.4% in oxygen uptake on a wet weight basis (h value) occurred in the adrenalectomized group. The parallel between the two increases is what we would expect if we assume that adrenalectomy produces no change in the enzyme: protein ratio. In the 4th experiment, protein nitrogen per dry weight of tissue was computed and found to show an increase of 17.8% above the control value. In the previous experiment, Q_{O_2} on a dry weight basis rose from 85.12 to 104.93, an increase of 23.2%. From these data we conclude that no change in the concentration of liver SDH-ase relative to concentrations of other liver proteins was produced by adrenalectomy.

It can be seen from the data (table 4) that some tissue hydration occurred under the conditions of adrenal insufficiency. This is shown by the 5.7% decrease in tissue solids in liver from the adrenalectomized animals. As in the previous experiment, there was a significant (12.9%) increase in the quantity of total nitrogen present in the liver tissue of the adrenalectomized animals when compared with the controls.

A comparison of the lipid content of the livers of the adrenalectomized and the control animals shows no significant difference when the quantities are expressed in per cent of wet weight of liver. If the average values for each are converted to per cent of total solids it is seen that in the adrenalectomized group the lipids represent 14.9% of the tissue solids and in the control group 13.1%. The difference is mainly accounted for by the decrease in total solids per unit wet weight in the adrenal insufficient groups. Furthermore, the proteins and fats together made up 74.1% of the total solids in the control animals, whereas they composed 86.8% of the total solids in the livers of the adrenalectomized animals. The remainder of the solids in each case consists of

non-protein nitrogenous compounds, inorganic salts and carbohydrates. Depletion of carbohydrate and of inorganic salts (Schwarz, '10; Silvette, '34; Marenzi, '38) may be responsible for the relative increase in protein and fat expressed as per cent of total solids.

These data justify the assertion that when enzyme measurements are made under circumstances in which changes in tissue constituents are seen, the enzyme concentrations must be expressed in meaningful terms. Adrenalectomy in this strain of rats results in relative increases in tissue fluid, protein nitrogen, non-protein nitrogen and, to a slight extent in lipids, as well as a relative increase in SDH-ase concentration. It is assumed that there is a relative decrease in carbohydrates and inorganic salt concentrations. Since the enzyme is presumably a protein, we believe that a decision as to whether its concentration has been increased or decreased should be made in terms of whether the ratio of the algebraic sum of its synthesis (or activation) and its destruction (or inactivation) to the similar algebraic sum of the synthesis and destruction of all other protein components changes or remains the same. SDH-ase changes after adrenalectomy, based on wet weight or dry weight of tissue are as meaningless as SDH-ase changes in terms of tissue fluid or lipid. It is, therefore, concluded that in order to give biological significance to expression of enzymatic activity, such expressions should be made in terms of tissue proteins, especially when changes in the relative concentrations of fat, carbohydrates and proteins occur.

SUMMARY

1. The SDH-ase activity of rat liver tissue under normal conditions and conditions of adrenal insufficiency was studied using a fortified substrate system. The activity was expressed in terms of oxygen uptake (a) per unit wet weight of tissue, (b) per unit dry weight of tissue, and (c) per unit of tissue nitrogen.

2. In the strain of rats used in this study, adrenalectomy resulted in an increase in oxygen uptake when expressed in terms of wet or dry weight of tissue, but no increase over the control value when expressed in terms of tissue nitrogen.

3. Adrenalectomy resulted in tissue hydration and in an increase in the concentration of tissue protein and non-protein nitrogen. The increase in protein nitrogen approximately paralleled the increase in oxygen uptake.

4. No significant change in liver lipid concentration in fresh tissue occurred under the conditions of adrenal insufficiency produced in this experiment.

5. The change in concentration of liver protein during adrenal insufficiency is possibly due to changes in the concentration of liver carbohydrates and inorganic salts.

6. It is concluded that there was no change in the rate of enzyme synthesis relative to the rate of synthesis of any other protein tissue constituents during periods of adrenal insufficiency.

7. When enzyme activities are measured under conditions where significant changes in relative concentrations of water, fat, carbohydrate and protein are seen, the enzyme activities should be expressed in terms of protein nitrogen.

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THE SIMULTANEOUS MEASUREMENT OF SEVERAL PARAMETERS OF EMBRYO HEART EXPLANT GROWTH IN VITRO[1]

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EIGHT FIGURES

In the first paper of this series by Signorotti, Hull and Kirk ('50) it was stated that the purpose of the investigation was to make extensive quantitative studies on growth and development of tissue in culture. In accordance with this plan, Hull and Kirk ('50a, b, c) studied the relationship of nucleic acid increase to the growth of the tissue, the incorporation of P^{32} into the nucleic acid fractions of the tissue, and the relationship of the total P^{32} uptake to the total nucleic acid increase. In addition, Boyer and Kirk have considered the relationship between the protein nitrogen increase of embryo heart tissues grown *in vitro* and their uptake of radioactive phosphate from various liquid media. The present paper adds several more correlations as determined in the same general type of system. These include the increase in the surface area of the explanted tissue as measured in the same experiments in which the total nitrogen content of the washed 6-day cultured cells, the day by day P^{32} uptake and the pH values of the used media were also determined. In this case, however, the measurements were undertaken primarily to establish a series of reference "growth indices" to be used for comparative purposes in testing the growth effects on tissue of fractions isolated from embryo extract. This latter work will be reported in a later paper of this series.

[1] Aided by grants from the American Cancer Society recommended by the Committee on Growth, and the Committee on Research of the University of California.

EXPERIMENTAL

The culturing techniques used were similar to those described by Signorotti, Hull and Kirk ('50) with the following exceptions: (a) Hearts from 11½-day chick embryos were used throughout; (b) only one-half heart was used per culture tube; (c) each half heart, after weighing, was cut into 8 approximately equal sized pieces which were aligned in two parallel rows of 4 each to expedite the area-tracing procedure; (d) 0.2 ml of plasma diluted 1 to 10 with Tyrode's solution were used per tube as the clot for supporting the explants; (e) after the original 3 ml charge of liquid medium was added, a gas mixture of 5% CO_2 in air was passed into each tube to adjust the pH; (f) all media were changed at the end of the second and 4th days of culturing, and the new media re-equilibrated with the 5% CO_2 mixture; (g) "blanks" were run simultaneously with each series of tissue-containing tubes, each blank tube receiving the same amounts of medium and the same gas treatment as the "tissue" tubes; and (h) for the experiments represented by figures 1–5, 6 tissue tubes and 6 blanks were run per set, and for those represented by figures 6–8, 5 tissue tubes and three blanks were used per set.

The "blanks" were prepared by placing 0.15 ml of plasma which had been diluted 1 : 10 and 0.05 ml of embryo extract in each tube. As this mixture coagulated, the tube was rotated so that the clot solidified in approximately the same position as that occupied by the clot in the tissue tubes.

Total nitrogen

The total nitrogen content of the cells was determined by a method modified from that used by Boyer and Kirk to determine protein nitrogen. At the end of 6 days of culturing, the liquid media were poured off and the tissues washed twice at 37°C. for one-hour periods with 0.9% NaCl, then defatted by treatment for 30 minutes at the same temperature with 25% chloroform in ethyl alcohol. One milliliter of concentrated sulphuric acid was then added to each culture tube,

and the contents were digested by heating the tubes in a sand bath until the contents became clear. Five drops of Superoxol (30% H_2O_2) were then added to each tube and the heating continued until the liquid became water white. Each digest was then transferred to a micro-Kjeldahl distillation unit of the Kirk ('36) type, and the nitrogen analysis carried out as recommended by that author. Each blank tube was analyzed in the same manner. Zero-time nitrogen values for uncultured tissue were also determined by preparing tissue tubes and blanks in the usual manner, followed by the washing, defatting and analyses as described.

P^{32} uptake

The amount of radioactive phosphorus taken up by the contents of each tissue and blank tube was determined at 24-hour intervals in essentially the same manner as described by Hull and Kirk ('50). In addition, each tube was counted at the end of the saline-washing step and at the end of the defatting step as the samples were being prepared for the nitrogen analysis.

Area increase

The change in surface area of the explants was determined by a modification of the method of Ebeling ('21) whereby the tracings made of the reflected explants were cut out and weighed on an analytical balance. Tracings were made at zero-time, and after the first, second, third, 4th and 6th days of culturing. The zero-time tracings were found to average 37.6 mg of paper per milligram of tissue, with a standard deviation of 4.6 mg. The reproducibility of the tracing weights was somewhat less with cultured tissues, since the colony edges were often poorly defined, particularly in systems containing only embryo extract, with no added horse serum. In this medium, the colonies were partially grown together after the 4th day, so further tracings were not possible.

pH of used media

The media from each set of tissue tubes were pooled at the time they were removed from the cultures. This took place at the end of the second, 4th and 6th days. The media in the blank tubes were similarly pooled at the same times. The pH values for each of these pooled media were immediately determined with a Beckman Type G glass electrode pH meter.

Materials used

The embryo extract used was prepared from 11½-day chick embryos (with the eyes removed) as described by Tompkins, Cunningham and Kirk ('47). The horse sera were commercial products from Cutter Laboratories in Berkeley, California, and from Microbiological Associates, Coral Gables, Florida. The horse serum ultra-filtrate used in the experimental work was obtained from the latter source, as was the dried plasma used for preparing the clots. Embryo extract ultra-filtrate was prepared by replacing the sterilizing disc of a Seitz filter with a circle of 300 weight plain transparent cellophane. After sterilizing the assembled filter, embryo extract was placed in the upper chamber and the unit was connected to a pressure line in a cold room at 4°C. The filtration was slow, the yield being only about 4 ml of ultra-filtrate per day.

A Tyrode solution containing extra glucose (4 g/l) and correspondingly lowered sodium chloride was used in making up all media. Radioactive phosphorus was obtained as phosphate solution from the Atomic Energy Commission Isotopes Division at Oak Ridge, Tennessee.

RESULTS

The P^{32} uptake and explant area increases are compared for tissues grown in 8 different media in figures 1 to 8. The areas are given in terms of milligrams of paper tracing per milligram of explanted tissue. The gross P^{32} count is given in terms of counts per second per milligram of explanted

tissue. In order to express the radioactivity taken up by clot blanks in the same units, the average gross count of the blank tubes of a set was divided by the average number of milligrams of tissue originally explanted per tube in the same set. This blank value was subtracted from the gross

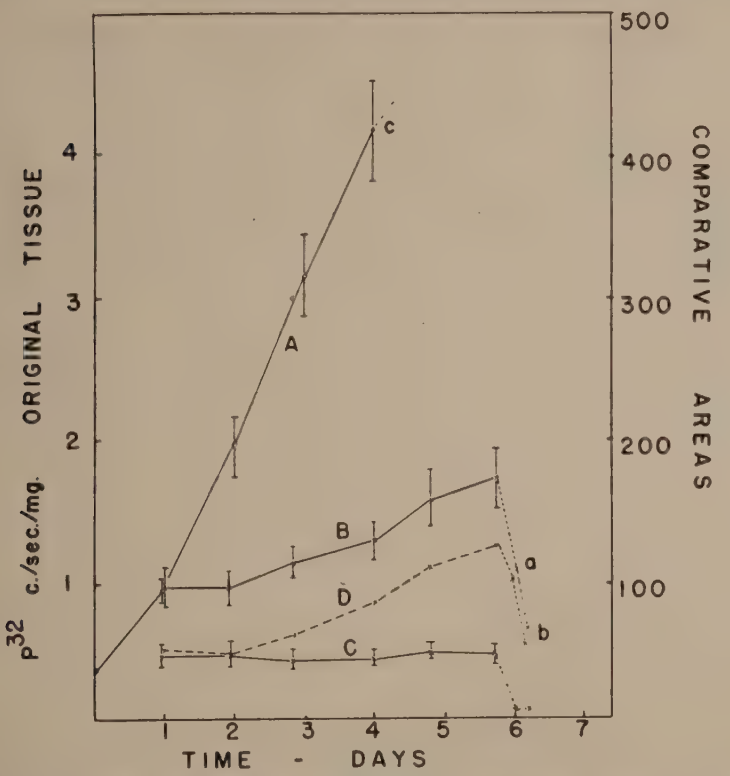

Fig. 1 P^{32} uptake and area changes. Medium: 20% embryo extract. A, areas; B, gross P^{32} count; C, blank P^{32} count; D, net P^{32} count. a, saline washed; b, defatted; c, colonies grown together.

P^{32} count per milligram to give the "net" P^{32} count shown on the graphs.

The dotted lines at the end of each of the P^{32} count curves indicate the drop in the radioactivity caused by the saline washing and the defatting procedures as the samples were being prepared for the nitrogen analyses. The vertical line

at each of the experimental points indicate the standard error of the mean for the values found for the individual tubes of each set. These errors are comparatively large in some cases for the gross P^{32} counts. This is primarily due to the fact that only 8 pieces of tissue were used per tube, so slight variations in their relative alignments from tube to

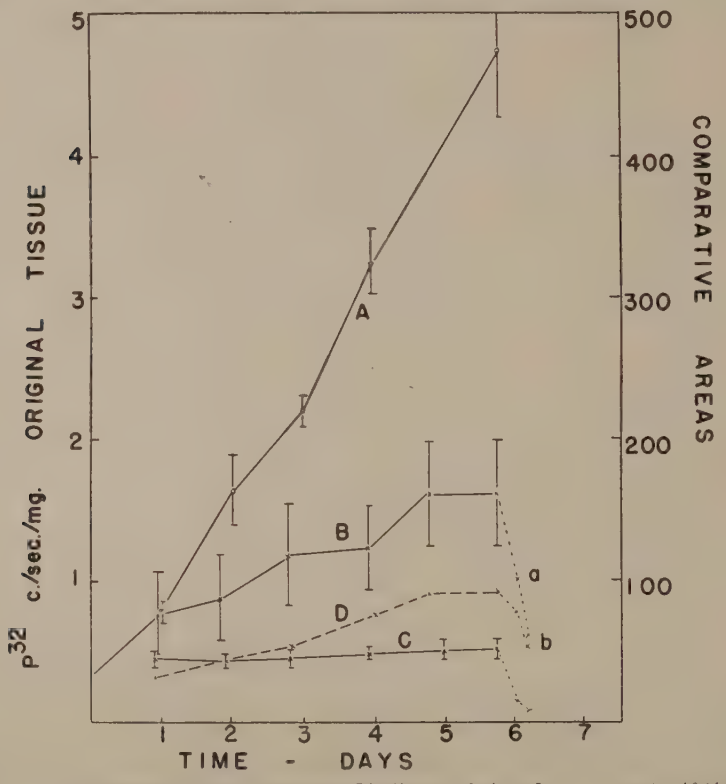

Fig. 2 P^{32} uptake and area changes. Medium: 20% embryo extract; 40% horse serum ("standard medium"). Symbols same as figure 1.

tube produced relatively large differences in the amount of radioactivity affecting the Geiger tube, due to the difference in the geometry of the system. It was felt that the magnitude of these errors was not of too great significance in this study, since the "growth indices," or relative values at different times were the criteria being established.

A mean of 9.9 μg of total nitrogen per milligram of washed, defatted uncultured ("zero-time") heart tissue was found as the mean of the values for a series of 12 analyses (standard deviation, 0.7 μg). Ten similarly treated blank tubes averaged 35 μg of nitrogen found for each of the tissue tubes, and the resulting difference was divided by the number of

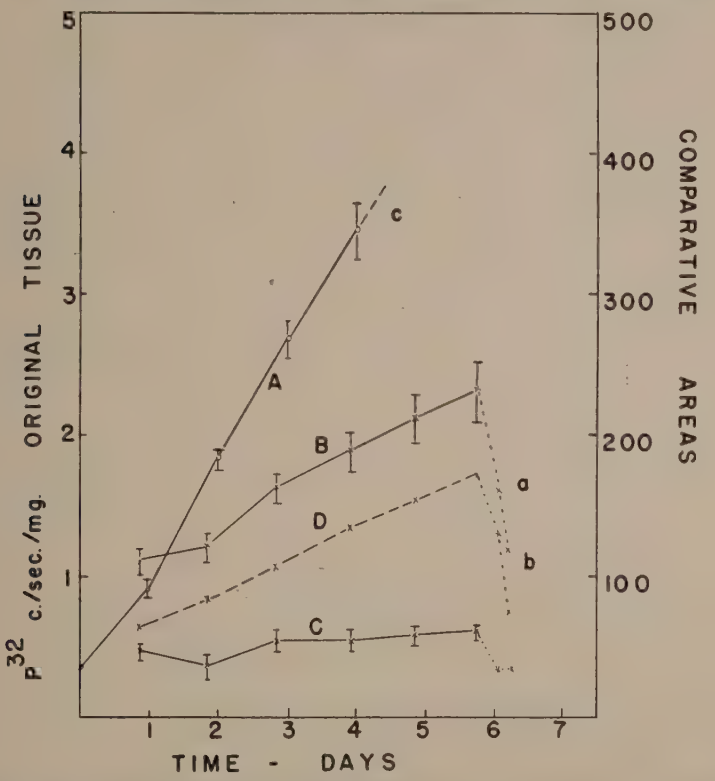

Fig. 3 P^{32} uptake and area changes. Medium: 20% embryo extract; 40% horse serum ultra-filtrate. Symbols same as figure 1.

milligrams of tissue explanted in the tube to give the "net" nitrogen value. The mean thus calculated from the 12 tissue analyses was 7.9 μg of "net" nitrogen per milligram of zero-time tissue (standard deviation = 0.6 γN).

The gross and net nitrogen values for tissues grown for 6 days in the 8 different media are summarized in table 1. The

(1:1) shown wherever embryo extract is mentioned refers to the fact that one volume of Tyrode solution was used to extract one volume of embryo pulp in preparing the extract stock, so that the resulting extract can be considered as actually being of only 50% strength.

The pH values for the various pooled media removed from the culture tubes are summarized in table 2.

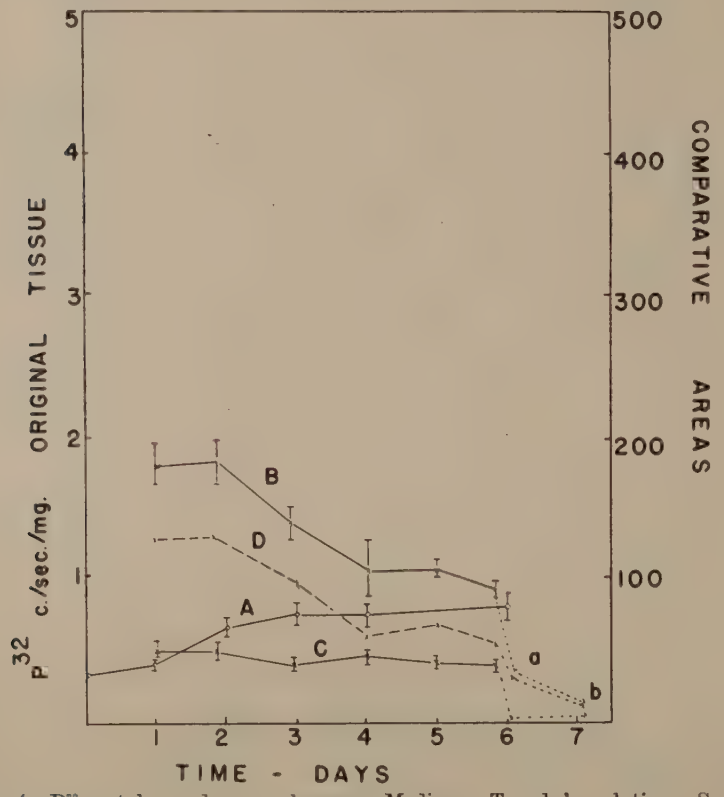

Fig. 4 P^{32} uptake and area changes. Medium: Tyrode's solution. Symbols same as figure 1.

DISCUSSION

As previously described, the purpose of this work was to establish a set of growth indices to serve as a frame of reference in evaluating the effects on cultures of various fractions isolated from embryo extract. These indices as calculated

were the ratios between the values of the observed measurements at various times in the culturing period, as for example, the ratios between the nitrogen content of the tissues after 6 days' culturing and at zero-time. A number of these indices are shown in table 3 for tissues grown in the 8 different media tested.

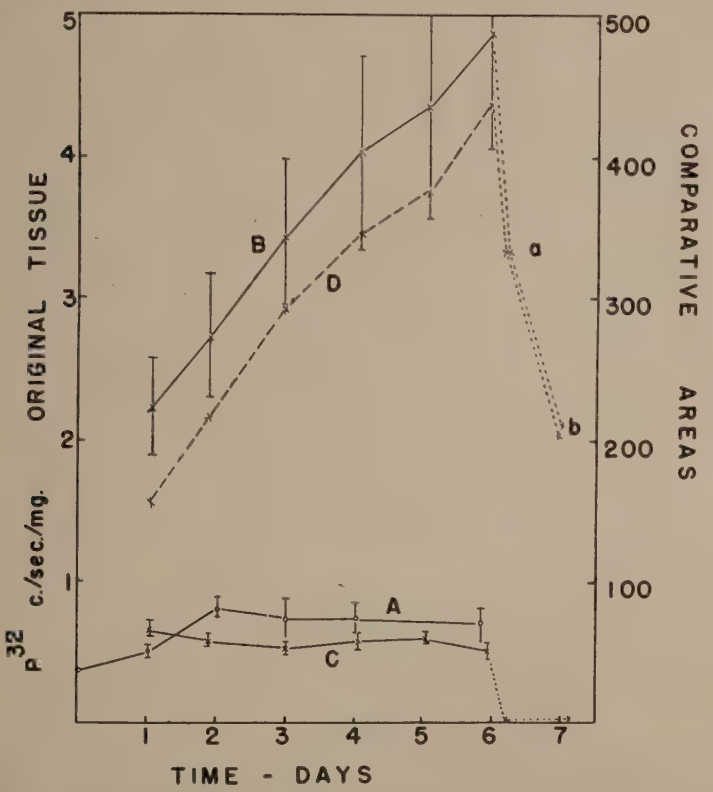

Fig. 5 P^{32} uptake and area changes. Medium: 40% horse serum. Symbols same as figure 1.

The first three media all contained embryo extract, and it will be seen that, with one exception, the magnitude of the indices calculated from the data of these runs is consistent with the idea that the tissue actually grew in these three media, whereas it did not in the remaining 5 cases. This agreed with the appearance of the cultures, although it is

true that the explants remained fresh looking and more alive in appearance in the ultra-filtrate media than in the remaining three cases, although they did not develop very much growth zone of cells.

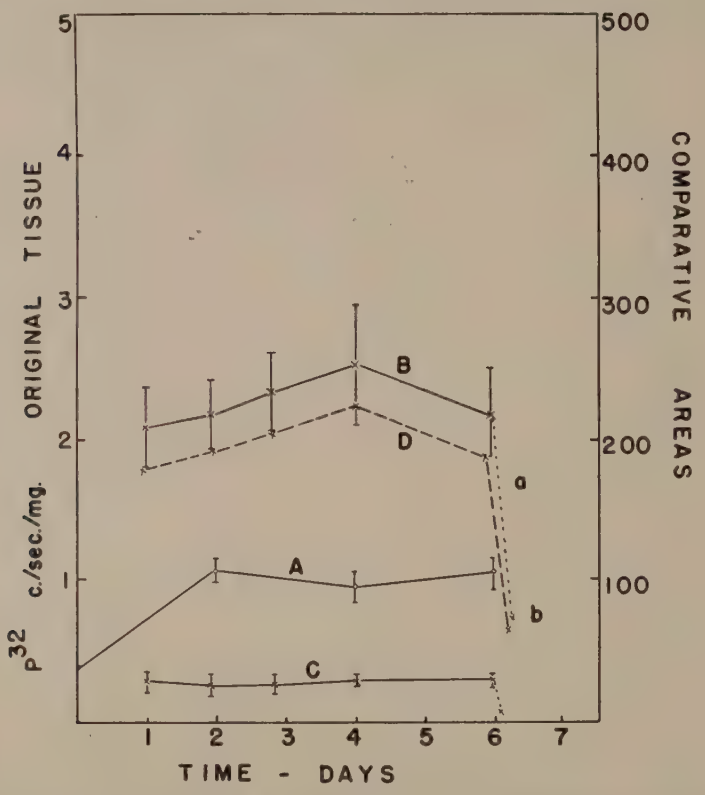

Fig. 6 P^{32} uptake and area changes. Medium: 40% horse serum ultra-filtrate. Symbols same as figure 1.

The anomalous index noted above occurred in the case of the P^{32} uptake observed for tissues grown in 40% horse serum alone. In this case, the radioactivity increase would indicate a substantial amount of growth, whereas the area and nitrogen change indicated that this was not the case. Tissues grown in serum from a second source did not show this contradictory behavior, and the P^{32} uptake was much

less. It was later learned that the first serum had come from an animal which was infected with a non-pathogenic strain of bacteria. The differences in the tissue behavior caused by this serum suggest some relationship to the experiments of

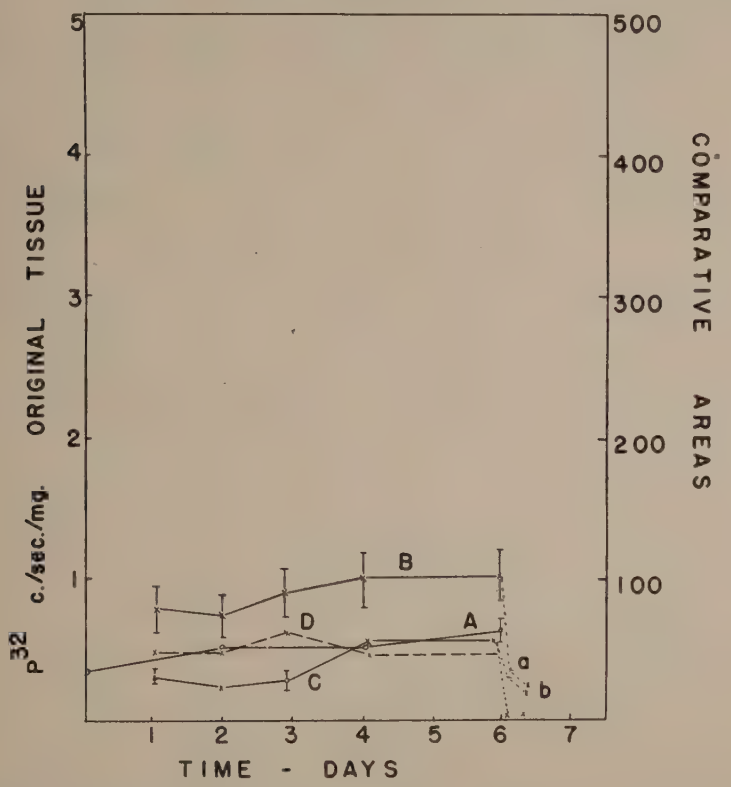

Fig. 7 P^{32} uptake and area changes. Medium: 20% embryo extract ultrafiltrate. Symbols same as figure 1.

Norris and Majnarich ('48a, b) in which they found that normal blood sera and sera from animals with tuberculosis increased the rate of proliferation of animal cell suspensions, whereas sera from animals with neoplastic growths or pernicious anemia caused strong inhibition of this activity. There might also be a more direct explanation in the observation of Laser ('39) that cells grown in serum showed more weight

increase than those grown in embryo extract, apparently due to an increased uptake of water in the former medium. This would account for the high P^{32} labelled inorganic phosphate in such cells, although, in such a case, it might be expected that the radioactivity would be markedly reduced

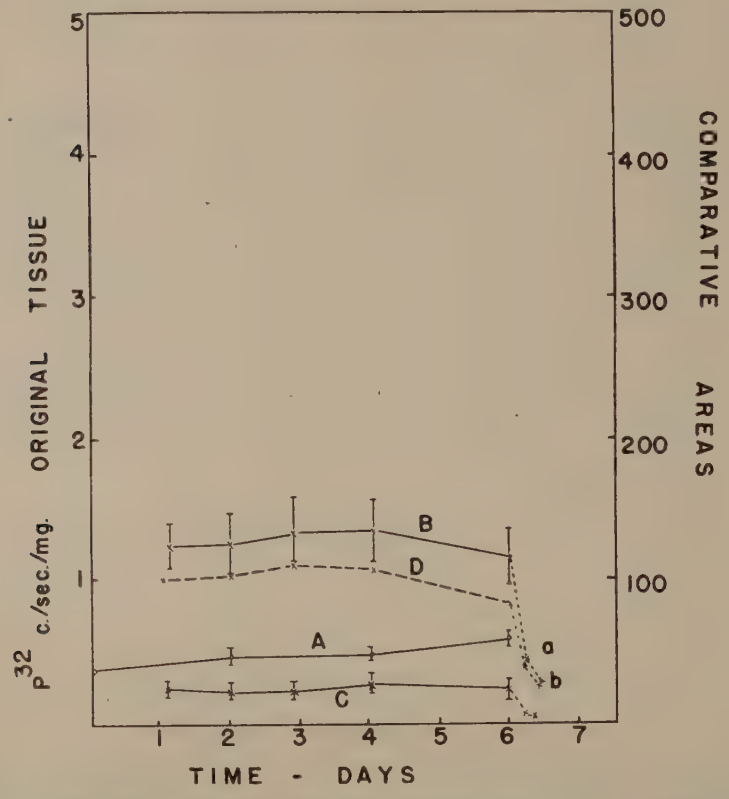

Fig. 8 P^{32} uptake and area changes. Medium: 20% embryo extract ultrafiltrate; 40% horse serum. Symbols same as figure 1.

by saline washing, whereas the relative loss observed was actually less than for tissues grown in Tyrode's solution. Willmer ('42) has suggested that the effect observed by Laser might be due to the formation of extra cytoplasmic growth in serum medium, or to excessive formation of intracellular matrices. If this last supposition were correct, and if such

TABLE 1

Total nitrogen analyses of washed and defatted six-day cultures grown in various media

MEDIUM	MICROGRAMS KJELDAHL N PER MG ORIGINAL TISSUE				
	Tissue tubes		Clot tubes		Net
	Value	Deviation	Value	Deviation	Value
20% (1:1) Embryo extract	28.2	6.0	17.6	1.4	10.6
20% (1:1) Embryo extract 40% Horse serum	18.6	3.4	5.8	0.8	12.8
20% (1:1) Embryo extract 40% HSUF[1]	17.5	1.3	7.0	2.6	10.5
Tyrode solution	7.8	1.7	2.2	0.8	5.6
40% Horse serum	9.6	1.8	3.3	0.7	6.2
40% HSUF[1]	7.2	0.8	1.0	0.6	6.2
40% Horse serum 20% EEUF[1]	6.7	0.4	2.5	0.8	4.2
20% EEUF[1]	7.8	0.4	1.8	0.7	6.0

[1] HSUF — Horse serum ultrafiltrate.
EEUF — Embryo extract ultrafiltrate.

TABLE 2

pH values for various media after 48-hour contact with embryo tissue or with plasma clot blanks

MEDIUM	pH VALUES OF MEDIA REMOVED:					
	First 2 days		Second 2 days		Last 2 days	
	Tissue tubes	Clot tubes	Tissue tubes	Clot tubes	Tissue tubes	Clot tubes
20% (1:1) Embryo extract	7.16	7.32	7.09	7.14	7.03	7.36
20% (1:1) Embryo extract 40% Horse serum	7.15	7.55	6.98	7.32	6.90	7.32
20% (1:1) Embryo extract 40% HSUF[1]	7.19	7.52	7.18	7.42	7.17	7.46
Tyrode solution	7.22	7.63	7.23	7.38	7.38	7.36
40% Horse serum	7.58	7.72	7.45	7.68	7.41	7.60
40% HSUF[1]	7.40	7.56	7.38	7.57	7.32	7.30
40% Horse serum 20% EEUF[1]	7.09	7.19	6.94	7.04	6.92	6.97
20% EEUF[1]	7.26	7.39	7.22	7.27	7.13	7.28

[1] HSUF — Horse serum ultrafiltrate.
EEUF — Embryo extract ultrafiltrate.

matrices were relatively high in phosphorus content, the rapid P^{32} uptake observed in this work might be explained. This would assume, of course, that this process was stimulated by some types of sera, but not by others.

Calculations were made of the percentage of the radioactivity removed from the tissues and from the blanks by the saline washing and defatting procedures. The only consistent trend observed was that a smaller portion of the

TABLE 3

Calculated ratios for use as indices of tissue growth

MEDIUM	P^{32} COUNT		NITROGEN		AREAS		
	6th day/1st day		6th day/0 time		2nd day	4th day	4th day
	Gross	Net	Gross	Net	0 time	0 time	2nd day
20% (1:1) Embryo extract	1.8	2.5	2.9	1.4	5.7	12.4	2.2
20% (1:1) Embryo extract 40% Horse serum	1.9	2.7	1.8	1.5	4.7	9.4	2.0
20% (1:1) Embryo extract 40% HSUF[1]	2.1	2.7	1.7	1.3	5.1	9.7	1.9
Tyrode solution	0.51	0.43	0.77	0.71	2.0	2.1	1.1
40% Horse serum	2.2	2.8	0.97	0.80	2.2	2.0	0.91
40% HSUF[1]	1.0	1.0	0.72	0.78	2.8	2.5	0.85
40% Horse serum 20% EEUF[1]	0.95	0.83	0.67	0.53	1.2	1.3	1.0
20% EEUF[1]	1.3	0.98	0.78	0.76	1.4	1.3	0.98

[1] HSUF — Horse serum ultrafiltrate.
EEUF — Embryo extract ultrafiltrate.

P^{32} was lost from those tissues grown in media containing embryo extract as compared with tissues grown in the 5 "non-growth promoting" media. This is partial confirmation of the assumption that most of the radioactive phosphorus taken up by the cells is going into a chemical form associated with the growth process, i.e., nucleoprotein, and that in general the P^{32} increase is a valid measure of growth. However, the results in the all-serum medium described above indicate

that P^{32} uptake alone cannot always be interpreted as representing true growth, and that it should always be used in conjunction with some other confirmatory testing method.

The data of table 3 confirm previous findings (Boyer and Kirk, '52) indicating that application of the term "maintenance medium" to liquids such as Tyrode's solution, horse serum, ultra-filtrates, etc., is not precisely correct, inasmuch as the explants are not completely "maintained," as indicated by the loss of total nitrogen. Similarly, the use of Tyrode's solution or other physiological salines as control media is dangerous, inasmuch as the experimental explants are then being compared to a dying tissue, and as a result, what might appear to be a favorable difference between the two actually is only a measure of the difference in the rates at which the tissue is dying in the two systems.

SUMMARY

1. Data have been presented showing the increase in surface area, the uptake of radioactive phosphate from the liquid medium, the increase in total nitrogen and the effect on the pH of the liquid medium of chick embryo heart explants grown in 8 different liquid media.

2. Growth indices have been calculated from these data to use for comparative purposes in testing the effects of unknown growth substances on cultured tissues.

3. These indices, in general, strongly support the belief that a tissue extract must be present in the liquid medium if true tissue growth is to occur.

4. An anomalous uptake of radioactive phosphate by tissues grown in certain serum media has been observed. This would seem to indicate that P^{32} uptake by itself should not be used as the only criterion of tissue growth, although its general validity and convenience still makes it a valuable tool if used in conjunction with some other confirmatory test for growth.

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CHANGES IN LIGHT SCATTERING ACCOMPANYING ACTIVITY IN NERVE

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SIX FIGURES

As compared to the studies of electrical, metabolic and thermal parameters of function in nerve, relatively little has been done explicitly to characterize activity in terms of ultrastructural changes which may occur when the cell goes from the resting to the active state and then recovers again. The reasons for this disparity stem from the facts (1) that the propagated disturbance is short lived, (2) that it probably produces chemical and ultrastructural changes which are at or beyond the limits of detection by orthodox methods, and (3) that at any region on the axone many of the changes which it produces are probably more or less completely reversed as the impulse sweeps by. The indirect method of studying chemical and structural changes under spatially separated polarizing electrodes, in an attempt to bypass these difficulties, based on the assumption that the impressed potentials may, to a degree, mimic certain aspects of the cathodal and anodal phases of the propagated disturbance, has been used in this laboratory, but with the understanding that any extrapolation to the propagating impulse would be hazardous and could be misleading. While one finds changes occurring under such electrodes, the insensitivity of detector devices used in the past has required high voltages, relatively intense currents and long times to make them detectable, and since

[1] This work has been aided in part by a grant from the United States Public Health Service, Neurological Diseases and Blindness Institute, and in part by a grant from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

such procedures usually render the nerve irreversibly inexcitable one is working with highly unphysiological states (Tobias, '51). Throughout the course of such work, therefore, there was the hope that one might develop techniques sensitive and rapid enough, and of high enough resolving power, so that structural changes at a sub-microscopic level could also be sought. With this in mind, experiments to use optical techniques were being planned when Hill and Keynes ('49) reported that the "opacity" of the crab nerve (Carcinus maenas) changes with activity. Subsequently it was reported by Hill ('50b) that the isolated giant axone of the squid undergoes diameter changes with activity, and it was suggested that both the "opacity" and diameter changes may be associated with transsurface ion movements favoring cellular-extracellular water shifts during activity (Hill, '50a, b).

Related experiments on light scattering by nerve are considered in this report. The word "scatter" is used with some misgiving. It will become clear that activity causes a change in the amount of radiation reaching a detector photocell from a nerve illuminated by a beam which is approximately at a right angle to the beam reaching the photocell. At this time, however, one does not certainly know how much of this change may be due to changes in molecular scattering, in reflection and reflectivity of larger units, in index of refraction, or perhaps even in fluorescence or photochemical absorption; no adequate experiments have yet been done with carefully monochromatized light or with polarized light. In other words, a completely adequate analysis of the change revealing its mechanism is not yet at hand. Therefore, one would like to use a word which is helpful in describing the experiments, but which does not at the same time connote interpretation. "Scatter," though not completely satisfactory, is being used for the time being.

The present communication is devoted primarily to three experimental points: (a) a confirmation of the fact that crab nerves change their light scattering properties as a function of activity, (b) the detection of a change, which is superficially,

at least, similar to that seen with the crab, in a vertebrate nerve, the frog sciatic with intact sheath, and (c) a demonstration that physical strain applied to the nerve conditions the optical change.

METHODS

The apparatus is similar to that of Hill ('50a). It is a balanced phototube device, the balancing being accomplished by controlling the light entering one cell. The nerve, partly submerged in its medium, is held in a beam of white light, and a fixed fraction of the amount of light scattered by it is measured.

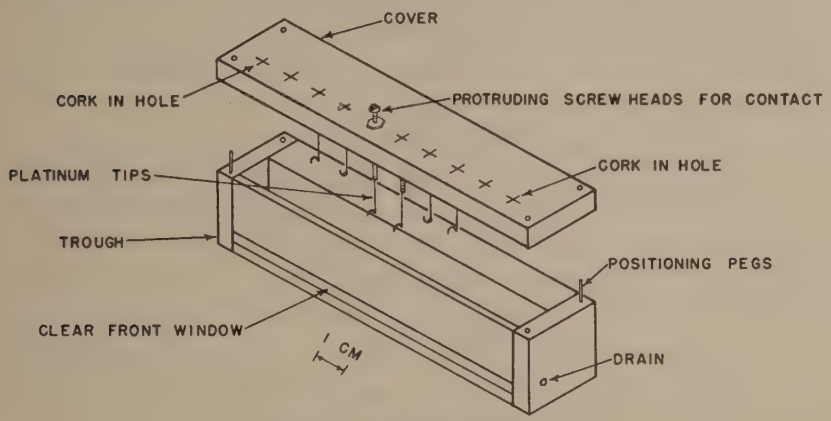


Fig. 1 Nerve chamber.

The lucite chamber (fig. 1) consists of a long, narrow trough of about 10 cm^3 volume with a cover about 6 mm thick to which the nerve supports are attached. The cover can accurately be positioned and held fast by screws.

The electrodes and supports are made of 0.8 mm platinum wires soldered into holes drilled into the ends of No. 4–36 machine screws. These wires are bent so as to cradle or straddle the nerve as needed, and such units are screwed through the cover and held fast by check nuts. Easily removable, modifiable and replaceable, this arrangement has proved convenient. Silk threads tied to the nerve ends are sewn through 1 mm holes containing compressed cork near

each end of the cover. Stretch on the nerve may be varied by pulling on the protruding threads which are clamped by the corks to prevent release. Electrical connections are made by clipping on to the protruding screw heads.

Placing the cover on the trough positions the nerve in it. The usual arrangement is to have the two central supports lower than the two at each end so that the two central ones alone extend below the solution in the trough. The trough is painted black except for two strip windows, one low down in front and one on the bottom. The low, submerged part of the nerve is held behind the front unpainted strip and over the bottom one. Light enters the front window to impinge on the nerve which deflects some of it through an approximately right angle to emerge through the bottom window. The chamber is clamped to a housing with the bottom window over a 1 mm slit below which a photocell is fixed (fig. 2).

The photoelectric device is diagrammed in figure 2. The light source is a rigidly mounted, 6 volt, 32 candlepower lamp positioned with the filament horizontal and parallel to the nerve. The light emerging from the "front" is focused by a 16 mm microscope objective to give a rectangular spot of light approximately 1 mm high and 2 mm wide where it illuminates the nerve. With the usual position of the nerve the detector photocell accepts a solid angle of about 0.5 steradian. The housing containing the detector photocell moves along brass rails parallel to the incident light beam and can be adjusted by a screw to focus the light spot on the nerve. The microscope objective may be slid up or down on the lamp housing to center the spot of light on the nerve. The light emerging from the "rear" of the lamp first passes through several sheets of white paper forming a neutral density filter which can be used as a coarse adjustment, and then through a Gaertner, adjustable spectroscope slit (500 mμ per revolution, 2 mm open full) into the balancing photocell.

The phototubes used to date have been red-infrared sensitive (RCA 917 and 919). The maximum response occurs at

about 8000 A.U. falling to approximately 20% at 4500 A.U., the stated sensitivity being 20 microamperes per lumen. Absolute light values appearing on the graphs are based on the manufacturer's statement of sensitivity. The phototubes are

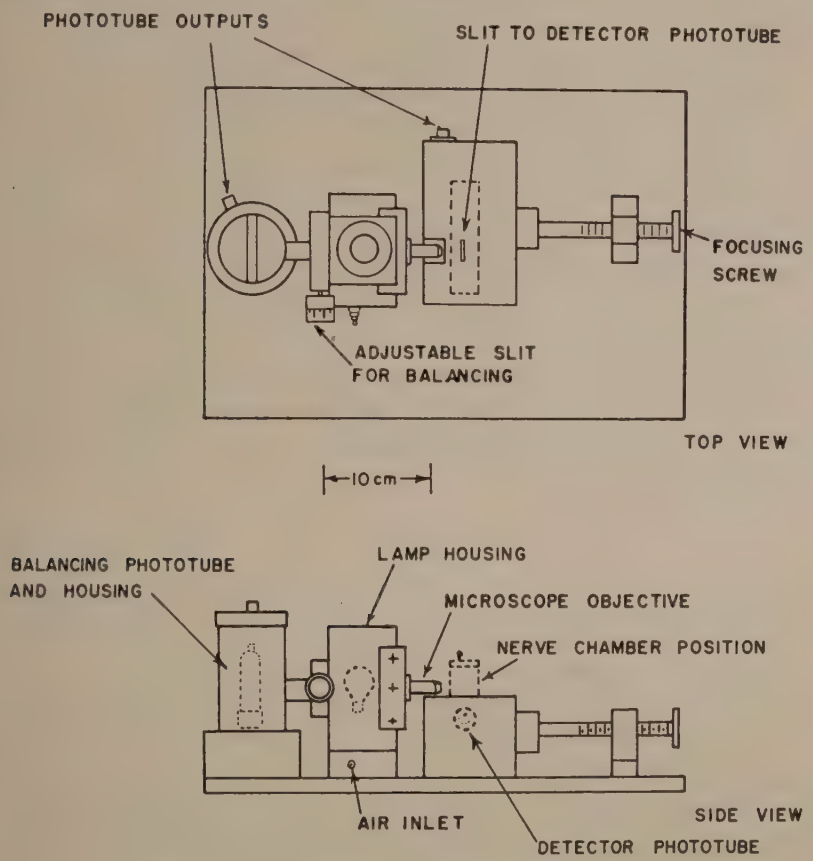


Fig. 2 Photoelectric device.

connected back-to-back with a common load resistor, stepwise variable to present different sensitivity scales. The output of the photoelectric system is led to a cathode follower (1000 meg. d.c. input res.) the output of which has most frequently been read on a 0.0073 microamp. per mm. galvanometer

at 5–10 sec. intervals and recorded by hand. Continuous recording was obtained by amplifying the cathode follower output with a differential d.c. amplifier leading into a dual beam oscilloscope. Recording, in these cases, was done photographically, the optical response being displayed on one beam of the oscilloscope, the stimulus on the other. Recordings have also been made with an ink-writing milliameter.

Equilibration of about 20 minutes has usually been enough to reduce instrumental drift to a small fraction, less than 1%, of the resting drifts usually encountered when the biological system is added. After a three-hour warm-up period, instrument drift rate was measured with a metallic reflector replacing the nerve. The unbalanced system had a drift rate of -0.011% per minute; with the balancing photocell connected and adjusted, as is the case in all actual experiments, the drift rate was $\pm 0.002\%$ per minute.

In all experiments either filtered sea water or normal frog Ringer has been used. The solutions were not bubbled either with air or oxygen.

RESULTS

When nerves are put in the chamber the scattered light shows an intensity drift (table 1, figs. 4 and 5). This may last for hours, frequently being rapid at first and then slowing down, rarely reversing spontaneously. Using Carcinus nerves in sea water, mounted with minimal stretch, such resting drift may be toward increased or decreased scattering in about equal numbers. With spider crab nerves similarly mounted the resting drift has been more frequently toward increased scattering (table 1). Whatever the cause of such drift, whether it be due to thermal, osmotic, chemical, mechanical, etc., changes, it is, as will be shown, susceptible to modification in terms of the amount of stretch and distortion applied to the nerve.

Secondly (table 2), in a number (14 nerves, 64 stim. periods) of early experiments with Carcinus nerves, though activity usually produced changes in scattered light, it was not pos-

TABLE 1

Effect of stretch on prestimulation drift in intensity of light scattered by crab nerve

ANIMAL	EXPERIMENTS DONE BEFORE EFFECT OF STRETCH WAS KNOWN			MINIMAL STRETCH			INCREASED STRETCH		
	No.	The scattering increased with time	The scattering decreased with time	No.	The scattering increased with time	The scattering decreased with time	No.	The scattering increased with time	The scattering decreased with time
Carcinus	9	3	6	14	7	7	9	0	9
Spider crab	8	8	0	16	15	1	8	1	7

TABLE 2

Effect of stretch on type of light scattering change produced by activity

ANIMAL	EXPERIMENTS DONE BEFORE STRETCH EFFECT WAS KNOWN						MINIMAL OR MEDIUM STRETCH						INCREASED STRETCH					
	Total no.		Response type [1] (%)				Total no.		Response type [1] (%)				Total no.		Response type [1] (%)			
	N [2]	S [2]	1	2 & 3	4	5	N [2]	S [2]	1	2 & 3	4	5	N [2]	S [2]	1	2 & 3	4	5
Carcinus	14	64	30	7	3	60	14	44	80	11	7	2	9	44	18	6	16	60
Spider crab	11	62	99	1	0	0	15	54	100	0	0	0	8	34	62	24	15	0

[1] Response type number refers to curves diagrammed in figure 3.
[2] N refers to number of nerves; S refers to number of stimulation periods.

Carcinus stimulations were at 31 pulses per sec. for 10 secs. in 32 cases, and at 31 per sec. for 5 secs. in 20. In the remaining trials frequency was from 1.5–87 per sec. and total duration was from 3–120 secs.

Spider crab stimulations were at 31 pulses per sec. for 10 secs. in 55 cases, and at 31 per sec. for 5 secs. in 86 cases. In the remaining trials frequency was at 31 per sec. and total duration was from 2–20 secs.

sible to foretell to which of the response types diagrammed in figure 3 the change would correspond. The curves in figure 3 are generalized and represent types of response to repetitive stimulation similar to that indicated in table 2. Most commonly this nerve responded in the fashion shown by curves 1 or 5, less frequently as shown by curves 2 or 3, and least often as shown by type 4. The effect of stimulation therefore was to produce a variety of types of change. Using

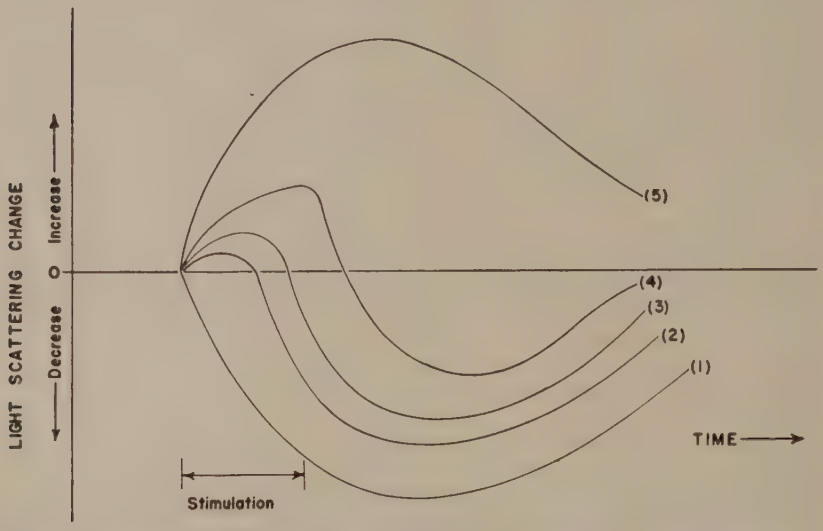


Fig. 3 Generalized response types. (N.B. not intended to imply temporal or geometric precision.)

spider crab nerves (11 nerves, 62 stim. period) (fig. 6) or frog nerves (6 nerves, 36 stim. periods, 64–430 pulses per sec. for 400 secs.) (fig. 4) only the type 1 response was seen. In addition to the inconstancy in response type, all three types of nerve showed considerable quantitative variability within any given response type, and even individual nerves varied in their responses from time to time.

Since Hill and Keynes ('49) found no detectable change with frog nerve, with "prolonged" stimulation at rates up to 200 per second, it is worth noting that a change has been seen

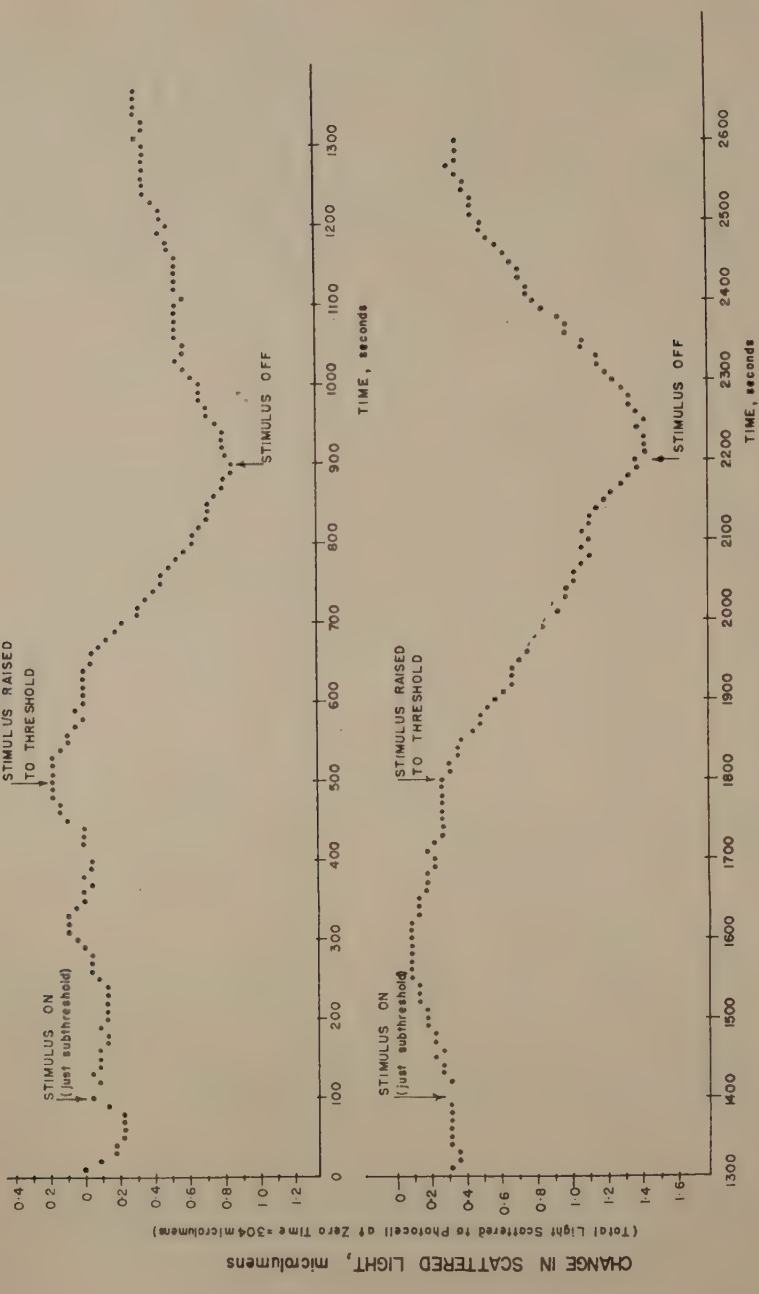
in the present experiments. The minimal amount of activity necessary to produce a detectable response is not yet known. Because of the large amount of connective tissue and myelin in the frog sciatic, and in these experiments the sheath was intact, it was anticipated that no change would be detected, but it has convincingly been seen during 36 stimulation periods using 6 different nerves. As in the case of the crab experiments, however, in some nerves it has not been seen at all even though apparently normal impulses were being conducted. Figure 4 is a graphical representation of two such responses from one nerve. It is of interest to know that a change similar, though not yet proven to be identical, to that seen in crab nerve also occurs in a vertebrate nerve.

The time course of such a phenomenon is important for interpretation. Later experiments (see below) suggest that a greater degree of precision may be expected on this point, but these earlier experiments have contributed little of a detailed sort. Nor does one yet know whether the optical change per axone reaches a plateau with continued stimulation. Two matters contribute to these difficulties: (1) it is not possible, with galvanometric display and manual recording to resolve very rapid changes, and (2) in the crab nerve certain fibers drop out in time, others fire sporadically, etc. Two facts, however, seem demonstrated: (1) the scattering change outlasts the stimulation period, and (2) the nerve recovers toward the original scattering level after stimulation is stopped (figs. 4, 5, 6). In general, these findings are similar to those of Hill.

The variability of the response has made it difficult to plan experiments. Therefore, causes of the variability have begun to be sought. Of the factors which could contribute it was suspected that the amount of tension applied to the nerve might be important.

Effect of combined stretch and distortion

In these experiments when the nerve was stretched it was also bent more strongly over the platinum supports and



(Stimulation frequency, 430 per second)

Figure 4

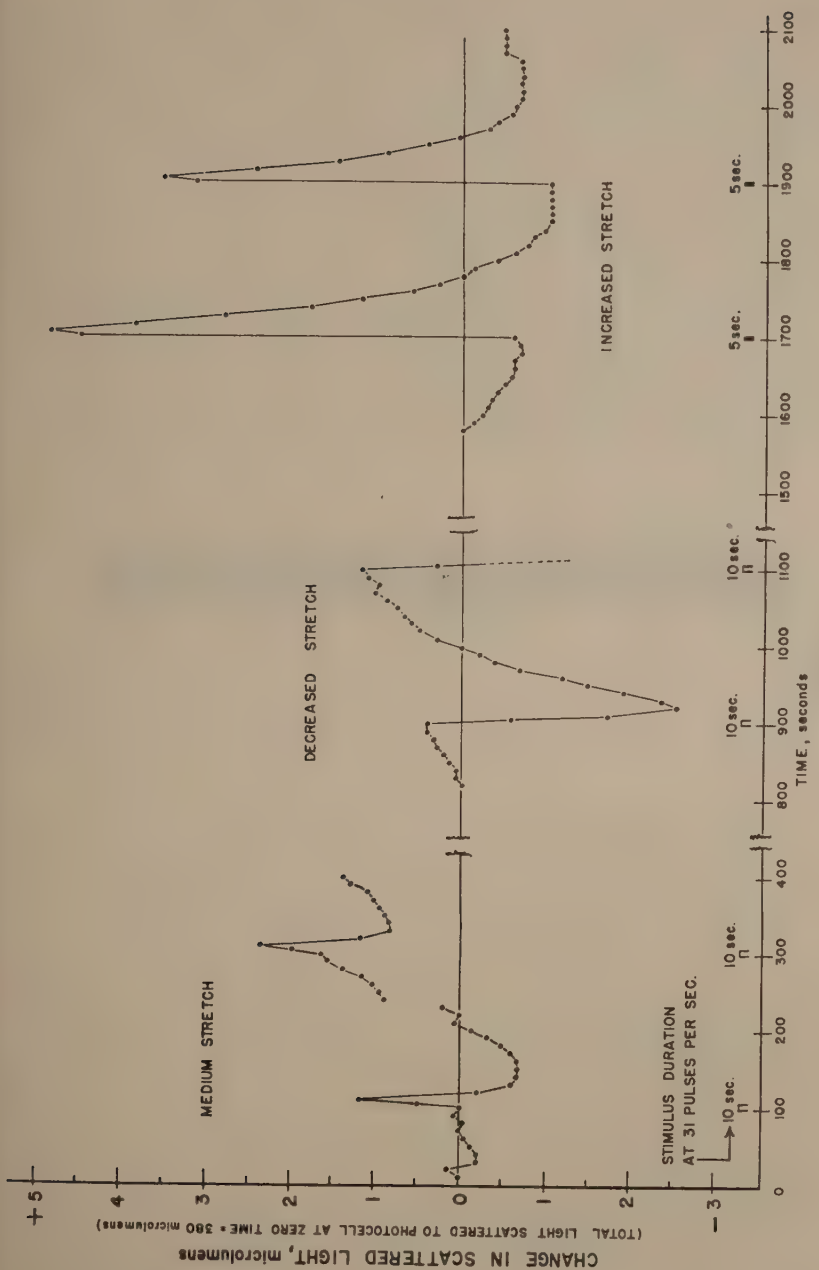

CHANGE IN SCATTERED LIGHT, microlumens
(TOTAL LIGHT SCATTERED TO PHOTOCELL AT ZERO TIME = 380 microlumens)
+5
4
3
2
1
0
1
2
3
MEDIUM STRETCH
DECREASED STRETCH
INCREASED STRETCH
STIMULUS DURATION
AT 31 PULSES PER SEC.
10 sec.
10 sec.
10 sec.
10 sec.
5 sec.
5 sec.
0
100
200
300
400
800
900
1000
1100
1500
1600
1700
1800
1900
2000
2100
TIME, seconds

Figure 5

electrodes and therefore compressed at these points. Whenever such treatment was sufficient to block, the experiment was discarded for the purpose of this communication. Satisfactory experiments to resolve these separate components, tension, bending and local compression have not yet been achieved.

The resting drift was usually found to change as a function of such treatment. This is shown in table 1 and figure 5. Carcinus nerves are shorter and more delicate than spider crab nerves and are therefore more subject to inadvertently applied stretch during mounting. In experiments done before the stretch effect was known and in which, therefore, stretch may have been accidentally applied, the resting drift, before

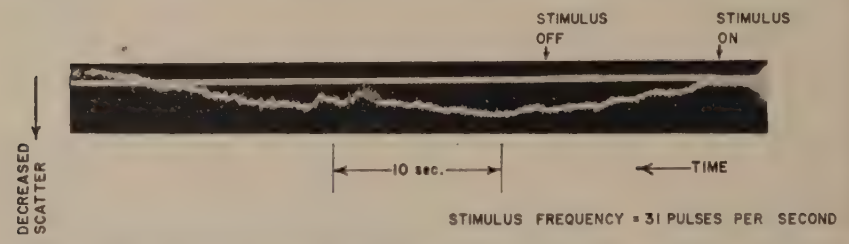


Fig. 6 Oscillographic recording of scattering change in spider crab nerve (moving film).

any periods of stimulation, was most often toward decreased scattering; even when a conscious attempt was made to have the nerves on minimal stretch the drift was toward increasing or decreasing light scattering in about equal numbers. However, when 9 such nerves were deliberately stretched the light scattering in all cases progressively decreased. Spider crab nerves mounted loosely show, predominantly, increasing scattering with time. As may, however, be seen from table 1 these nerves too under tension showed a shift toward a progressive decrease in scattering. Whatever the basis for the resting drift may be, therefore, it appears to be influenced by this procedure.

It was also found that the change which occurs with activity is modifiable by such treatment. Thus (tables 2 and 3 and

fig. 5), under low tension activity produces predominantly, though not always purely, a decrease in light scattering, whereas under stretch the change is shifted toward increased light scatter. Figure 5 is a graph showing the effect of stretch on the scattering response in a Carcinus nerve. This is not a composite graph, all the responses being exhibited by the same nerve in the order and at the time intervals indicated. In this case the direction of the prestimulation drift, the direction of the response to stimulation, and the magnitude of the response were all changed by stretch. It is also seen that recovery occurs, and that the scattering can continue to increase for a time after stimulation ceases. The sudden elevation of the baseline during the medium stretch period was probably due to mechanical disturbance of the nerve such as jarring. Only the start of the second response during minimal stretch is shown, because the response was so large that the galvanometer scale, at the sensitivity being used, was not enough to show it. Actually, for each of the three stretch conditions three responses were recorded, all qualitatively like those shown. The third in each group has been omitted.

One may say then that the quantitatively dominant effect of activity in these Carcinus nerves has been to reduce light scattering when minimally stretched and distorted and to increase it when such stretch and distortion are increased. It is not unlikely that this phenomenon is to a degree responsible for the variability of response seen in early experiments (tables 2 and 3) and previously reported by Hill ('50a).

Table 3 shows a breakdown of the data describing the relation between response type and stretch. This allows one to seen how many of the responses grouped in table 2 were obtained with individual nerves.

It is worth pointing out here that with Carcinus nerve on stretch a short-lived increase in scattering has convincingly and repeatedly been seen and recorded with a single shock. Hill also reported an effect of single stimuli. This, plus the fact that stretch often intensifies the change (fig. 5), makes

TABLE 3

Effect of stretch on type of light scattering change produced by activity[1]
(Breakdown of data summarized in table 2)[2]

CARCINUS												SPIDER CRAB											
Before stretch effect known				Min. or med. stretch				Increased stretch				Before stretch effect known				Min. or med. stretch				Increased stretch			
Actual number of responses of types indicated												Actual number of responses of types indicated											
1	2 & 3	4	5	1	2 & 3	4	5	1	2 & 3	4	5	1	2 & 3	4	5	1	2 & 3	4	5	1	2 & 3	4	5
0	0	0	5	4	0	0	0	1	3	3	0	13	0	0	0	5	0	0	0	2	0	0	0
0	0	0	6	3	0	0	0	0	0	1	2	6	0	0	0	3	0	0	0	0	0	5	0
8	1	0	0	3	0	0	0	0	0	0	3	9	0	0	0	4	0	0	0	3	0	0	0
4	1	0	0	3	0	0	0	0	0	0	3	10	0	0	0	4	0	0	0	1	8	0	0
0	0	0	4	4	0	0	0	7	0	0	0	5	0	0	0	3	0	0	0	2	0	0	0
0	0	0	6	4	0	0	0	0	0	3	0	1	0	0	0	2	0	0	0	1	0	0	0
1	1	1	5	3	0	0	0					1	0	0	0	3	0	0	0	6	0	0	0
0	2	0	0	4	0	0	0					1	0	0	0	9	0	0	0	6	0	0	0
3	0	0	0	3	0	0	0					1	0	0	0	3	0	0	0				
0	0	0	5	0	3	0	0	0	0	0	13	1	0	0	0	3	0	0	0				
3	0	1	0	0	2	0	0					3	1	0	0	3	0	0	0				
0	0	0	2	0	0	3	1									3	0	0	0				
0	0	0	3	2	0	0	0	0	0	0	3					3	0	0	0				
0	0	0	2	2	0	0	0	0	0	0	2					3	0	0	0				
																3	0	0	0				

[1] Notation as in table 2.

[2] Each horizontal line in this table refers to one nerve.

it somewhat more hopeful that the temporal relation of the optical response to the electrical change (spike) might more precisely be determined.

A second environmental parameter which conditions the optical change with activity is the osmotic pressure of the medium. Hill ('50a) found that hypotonicity can favor the appearance of increased scattering with activity. This effect has also been seen during the present experiments. Therefore, both stretch and hypotonicity seem to favor the same end result in terms of this phenomenon.

Since hypotonicity and stretch might summate, and since stretch did not convincingly shift the response in spider crab as it did in Carcinus nerve (tables 2 and 3), an attempt was made to dilute sea water (simple addition of distilled water) to a point where it would not alone reverse the response in the spider crab nerve, but at which osmotic pressure it might allow the stretch effect to become apparent. The results of such experiments have been suggestive only. Thus, four spider crab nerves on low tension in normal sea water all showed pure scattering decreases (curve 1, fig. 3) during three stimulation periods for each (31 pulses per sec., 5–10 sec. duration). Using these same nerves in 97, 95 or 85% sea water, but without increasing stretch, 8 out of 11 (73%) periods of stimulation produced small initial increases in scattering followed by decreases (curve 2, fig. 3); the remaining three stimulations produced pure decreases (curve 1). When stretch was now applied, 13 out of 17 (77%) periods of stimulation showed a shift toward increased scattering responses, and 8 of these were of the type (curve 5) characterized by large and pure increases in scattering. The 4 stimulations which failed to show any shift were all with one nerve. The fact that 8 responses were rather typical of those seen with Carcinus nerves on stretch, whereas, in no case at any other time in these experiments has such a change been seen with spider crab nerves, suggests that hypotonicity and stretch may summate in this respect. It is

entirely likely that other variables also are involved, and this matter is not yet adequately studied.

The effect of hypertonicity was not deliberately studied in the earlier experiments. However, Carcinus crabs shipped to Chicago (packed in moist wood shavings and kept in the icebox at about 5°C.) gave erratic and atypical responses though electrical activity seemed about as usual. Frequently nerves mounted as loosely as possible in sea water (shipped in glass containers) showed only pure, large scattering increases with activity. Since the animals might have been dehydrated it was felt that the sea water might be effectively hypotonic for the nerves coming from a now hypertonic body fluid. Therefore, such nerves after exhibiting the increased scattering response were bathed for 15 minutes in sea water to which sucrose had been added (3.86 gm per 100 cm^3), and were examined in this medium. It was possible by such treatment to convert the optical response to a large and dominant decrease in scattering with activity. Under these circumstances, therefore, hypertonicity produced by adding sucrose has an effect apparently opposite to that of simple dilution. An increase in refractive index, produced by sucrose addition, may also play a role in this apparent reversal (see Hill, '50a).

Controls

Because of experiments on optical changes produced in nerve by electrical polarization (Tobias, '51), there has been concern lest the changes found with activity might be caused by current spread from the stimulating electrodes. Hill and Keynes ('49) presented quite convincing evidence that this is not the case. The present controls corroborate this point of view, and tend to support the argument that the change depends upon propagated activity.

The controls have been several: (1) The change is not dependent upon or influenced by whether the cathode or anode of the stimulating electrodes is nearer the region being observed (distance app. 1.5 cm). (2) If one stimulates just

above threshold the optical change occurs. If the stimulus is just below threshold it does not, and recovery can occur even though the stimulator be held just below threshold. (3) Nerves which for some reason fail to conduct do not exhibit the optical change even though stimulus strength and pulse duration be much above the usual threshold quantities.

TABLE 4

Relation between scattering change and amount of stimulation

(2 Carcinus nerves on increased stretch)

NERVE	TOTAL NUMBER STIMULI	STIMULI PER SEC.	DURATION OF STIMULATION	AVERAGE RESPONSE, ARBITRARY UNITS
			secs.	
1	10	1	10	2
	15	1.5	10	10
	20	2	10	16
	70	7	10	23
	140	14	10	72
	310	31	10	100
	640	64	10	116
2	5	1	5	2
	7.5	1.5	5	7
	10	2	5	22
	15	1.5	10	20
	20	2	10	39
	35	7	5	38
	70	7	10	116
	140	14	10	159

It is still possible, however, that some change initiated at the electrodes, but only when threshold in a conducting nerve is reached, as for instance a compression wave, is very rapidly transmitted along the nerve and might contribute to the effects noted.

Impulse conduction without detectable optical response

As noted earlier the optical changes seen with activity are quantitatively inconstant. It may, however, be well to repeat that in some experiments, though apparently normal spikes

are seen, the optical response has been completely undetectable. It is not possible immediately to conclude from this that the underlying change responsible for the optical effect is not a constant accompaniment of activity, since in these cases the optical change may somehow be masked. However, such experiments invalidate, for the time being at least, any claim that the underlying mechanism is in fact a constant accompaniment of impulse propagation.

DISCUSSION

The work of Hill and the presently reported experiments describe a phenomenon perhaps not yet well enough understood to warrant extensive interpretation.

The possibility that the optical changes may be due to intra-extracellular water shifts has been considered (Hill, '50a, b). It is, however, not yet clear whether some intrinsic change in colloidal ultrastructure as part of or in addition to that resulting from water movement may also contribute. The precise time course of the changes as a function of the action potential is not yet known; nor are the precise locus or loci involved well defined.

A suggestion made by Hill to account for enhancement of the increased scattering phase by hypotonicity is that due to a raised hydrostatic pressure in the swollen fibers water leaves during activity, the fiber shrinks and scattering is increased. Stretch and the sort of distortion to which the nerves have been subjected in these experiments also enhances the increased scattering phase and may also increase hydrostatic pressure. However, lacking information about the elastic properties of these nerves one can say little of a quantitative sort. Nonetheless, superficially at least, the effect of stretch and bending compression may fit in with Hill's hypothesis.

Any precise quantification of the changes involves a previously unmentioned consideration. Perhaps the decreased scattering phase is due to swelling of fibers as Hill suggests, though he is careful to point out that at this time one can

really only claim temporal correspondence rather than a causal relation. However, if the swelling produces a decreased scatter then this should be corrected for the fact that the swollen nerve will intercept more of the incident light beam, and this factor would tend to produce an increase in scattering by bringing a greater percentage of the incident beam into the system. Therefore the actual decreases in scattering may be greater than they appear to be. A similar reasoning of reverse order is applicable to the increased scattering phase.

While control experiments strongly suggest that the phenomenon is not due to events occurring at the stimulating electrodes, this also is not yet rigorously ruled out, since as mentioned earlier, the critical change may be initiated there, but only coincident with reaching threshold in a normal nerve. Precise timing, if it is possible, should aid in resolving this matter.

The effects of stretch and distortion suggest that birefringence measurements such as those made by Schmitt and Schmitt ('40) might profitably be repeated with axones on stretch. However, possible interference by scattering and shape changes would have to be taken into consideration.

No adequate work has yet been done with a monochromatized source.

Using these more sensitive light scattering techniques it may be possible to tell whether any of the phenomena suggested by polarization experiments exist in reality as concomitants of propagated activity.

SUMMARY

1. Spider crab, Carcinus and frog nerves show changes in light scattering as a function of activity. In the crab nerves such changes have been seen and recorded following single shocks.

2. Stretch, accompanied in these experiments by localized bending and compression, influences the direction and magnitude of the scattering change both in the resting nerve and

with activity. The quantitatively dominant effect of activity in Carcinus nerve is to decrease light scattering by a lax nerve, and to increase it by a nerve under stretch. This reversal phenomenon is much less marked in spider crab nerve, and, to date, has required special conditions to bring it out. Hypotonicity also favors increased scattering with activity and may be able to summate with stretch to produce this effect.

3. The scattering change induced by activity outlasts the activity. Following activity the light scattered by nerve returns toward the resting level.

4. Neither the precise time course of the changes nor the exact anatomical loci of the elements responsible for the optical response are yet known. Nor is it yet possible to claim that the mechanism underlying the optical change is a constant accompaniment of impulse propagation.

ACKNOWLEDGMENTS

The writers are grateful to Dr. Robert Moon of the Institute of Radiobiology for helpful conversations on technical problems connected with apparatus. Dr. Harry Grundfest of Columbia University kindly loaned a d.c. amplifier, double beam oscilloscope and Fairchild camera for preliminary experiments with high speed recording. The Newark Electric Co. of Chicago generously gave the laboratory a large quantity of electronic supplies. Mr. Eric Hebel of the Physiology Shop assisted with construction of apparatus.

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THE OXYGEN UPTAKE OF HUMAN CEREBRAL CORTEX SLICES AND THE EFFECTS OF SOME INHIBITORS [1]

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TEN FIGURES

Although the *in vitro* metabolism of mammalian brain has been extensively investigated, very little has been done with human material because of its limited availability (Quastel and Wheatley, '32; Elliott, '48; Elliott and Penfield, '48). However, our understanding of the metabolism of the intact human brain is continually enlarging due to the recent development of adequate *in vivo* methods for the measurement of cerebral circulation and metabolism. Further *in vitro* studies are therefore desirable for comparison with *in vivo* findings (Gibbs et al., '47; Kety and Schmidt, '48).

The fortunate availability of brain biopsy material from pyschotic patients over an extended period of time has made the present studies possible. Because the patients from whom the specimens were obtained were suffering from advanced mental disease, the tissue cannot strictly be called "normal" except in a histological sense as there was no organic brain disease present. These initial studies are concerned with oxygen uptake under various conditions and are of an exploratory nature. Further work is in progress using other techniques to expand and elucidate the results obtained by manometric methods.

[1] Aided by a grant from the National Institutes of Health, Bethesda, Maryland.

MATERIALS AND METHODS

All experiments were done on cerebral cortex removed from Brodman's area 9 during the course of prefrontal lobotomy operations. Histopathological studies indicated that the tissue structure was within normal limits even though some of the patients had undergone more than 150 electroshock treatments as well as other types of shock therapy. Results presented always include data obtained from tissue from several brains, intercomparison was possible because all specimens were obtained from approximately the same cortical area. To date, efforts to correlate various findings with the psychiatric diagnoses or with previous therapy have failed so there has been no attempt to segregate results in this report. Immediately after surgical removal the specimen was placed in an ice-cold beaker and transferred to a moist cold box (Fuhrman and Field, '44). Adhering meninges were stripped off with fine forceps and thin slices of cortex were cut with a razor blade and template (Crismon and Field, '40). Approximately 40 mg samples of tissue were weighed on a micro-torsion balance and placed in chilled Warburg flasks containing 1–3 ml of Ringer's solution. The flasks were gassed with oxygen and allowed to equilibrate for 10 minutes at 37.2° before making the initial reading. The suspension medium contained 0.145 M NaCl, 0.004 M KCl, 0.0016 M $CaCl_2$, 0.0011 M $MgSO_4$, 0.01 M Na_2HPO_4 buffer pH 7.35, and organic substrates where indicated. When calcium free media were desired the $CaCl_2$ was omitted. The usual run lasted for three hours, readings being made every 15 minutes. Q_{O_2} values are expressed on a wet weight basis since the amount of tissue obtained was usually not sufficient for the determination of wet-dry ratios. Additions were usually made from the flask side arms after a control period of 90 minutes but in some experiments agents were added immediately after the initial reading. Substrates and all inhibitors except cyanide were made up in fresh Ringer's solution or water just previous to each run. Cyanide concentrations were attained by using the proper mixture of KCN-KOH in the center wells

of the Warburg flasks according to the method described by Robbie ('48). Respiratory quotients were measured by the second method of Dickens and Seimer (Umbreit et al., '49) using Warburg flasks equipped with siamese side arms.

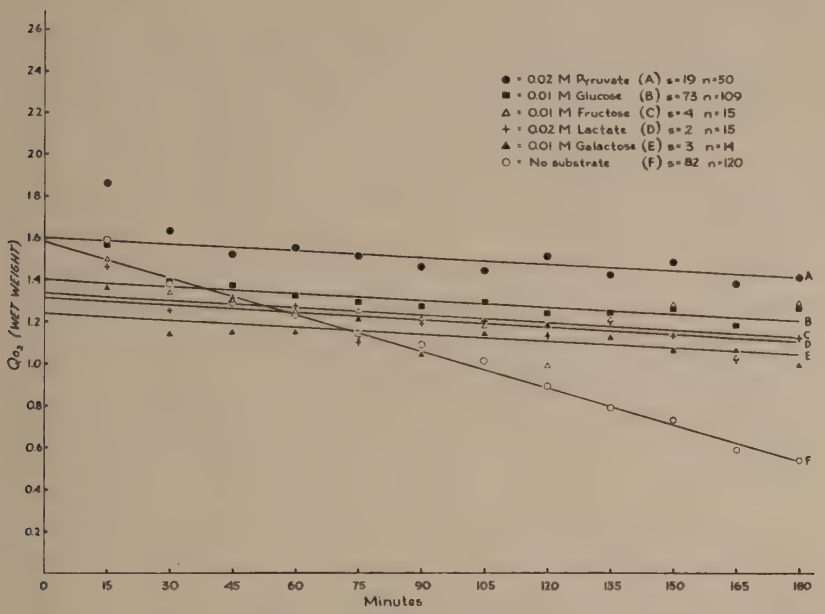

Fig. 1 The Q_{O_2} of human cerebral cortex slices in the presence of various substrates. In all figures (S or P) refers to the number of biopsy specimens and (N) refers to the number of vessels included in each curve.

RESULTS

The oxygen consumption of human cerebral cortex slices has been measured in the presence of various substrates including hexoses, lactate, pyruvate, succinate, and amino acids. In figure 1 the respiratory rates in the presence of glucose (0.01 M), fructose (0.01 M), galactose (0.01 M), lactate (0.02 M), and pyruvate (0.02 M) are compared with the endogenous Q_{O_2}. These substances maintained oxygen uptake for three hours with a fall in Q_{O_2} of only 12–15%. The glucose Q_{O_2} was 1.4 when extrapolated to zero time, about 58% of the value (2.4) for rat brain cortex slices determined under

identical conditions. This inverse relationship between Q_{O_2} and body weight agrees closely with that found by K. A. C. Elliott ('48) using a calcium-free phosphate buffered medium. The initial rate for pyruvate was 1.6 and the rate remained consistently higher than that for glucose. The initial rate for pyruvate was 1.6 and the rate remained consistently higher than that for glucose. At 90 minutes the pyruvate values were significantly higher than the glucose values ($p < 0.001$). Values for fructose and lactate approximated those for glucose but the galactose Q_{O_2} was slightly lower. Endogenous values during the first hour were similar to glucose values but actually fell off lineally with time. The rate at three hours was 0.54, 34% of the initial value. This was significantly lower than the 90 minute value of 1.09 ($p < 0.001$). Standard errors of the means were calculated for the 90 and 180 minute points of the pyruvate, glucose, fructose, and no substrate curves of figure 1. Since the number of vessels involved ranges from 15 to 120, these figures may be used to estimate the variability of the remainder of the data. The Q_{O_2}'s + S.E. of the mean at 90 and 180 minutes respectively are as follows: pyruvate, 1.46 ± 0.035 and 1.41 ± 0.029, glucose, 1.27 ± 0.017 and 1.26 ± 0.016, fructose, 1.21 ± 0.043 and 1.29 ± 0.082, no substrate, 1.09 ± 0.020 and 0.53 ± 0.018.

The effect of succinate on oxygen consumption is shown in figure 2. Concentrations ranging from 0.0009 M to 0.2 M elevated the Q_{O_2} above the endogenous level and it is apparent that the degree of stimulation was dependent upon the succinate concentration. In addition, the rate of oxygen consumption was not constant but paralleled the endogenous Q_{O_2} regardless of succinate concentration. A 40 fold increase in succinate concentration was required to raise the oxygen consumption above that produced by a concentration of 0.0009 M. Since additional amounts of succinate did not markedly increase the rate of oxygen consumption, 0.036 M apparently represents either the level at which the enzymes responsible for the oxidation of succinate are saturated or

the level at which the maximum rate of transport of succinate across the cell membrane is achieved. The Q_{O_2} with succinate was more variable than that with any other substrate, which may indicate the influence on the succinoxidase system of some factor such as age or previous treatment of the patients from whom the biopsies were obtained. This variability is reflected in the finding that the values obtained with 0.09 M succinate were higher than with 0.2 M succinate. The latter

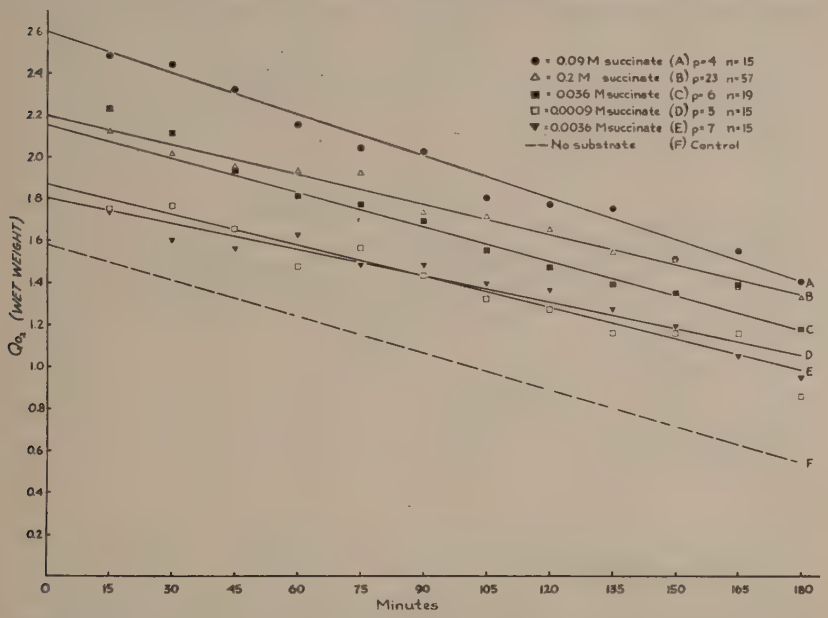

Fig. 2 The effect of increasing concentrations of succinate on the Q_{O_2} of human cerebral cortex slices.

curve was obtained from a larger series of specimens over a longer period of time. The differences, although appreciable in some cases, could not account for the concentration effect portrayed in figure 2 since the same results were obtained when two or more concentrations were used on tissues from a single biopsy.

The effect of amino acids on the Q_{O_2} was also tested since certain amino acids give rise to products known to enter the tricarboxylic acid cycle and contribute to cellular energy pro-

duction. In order to determine whether an amino acid mixture such as is present extracellularly could support human brain respiration, 0.4% vitamin free casein hydrolysate (Difco) was used as a substrate. This preparation supported the respiration of rat brain slices only in the presence of glucose. With human brain slices, however, the amino acid mixture alone maintained the Q_{O_2} above the endogenous level for three hours (fig. 3). The initial values approximated

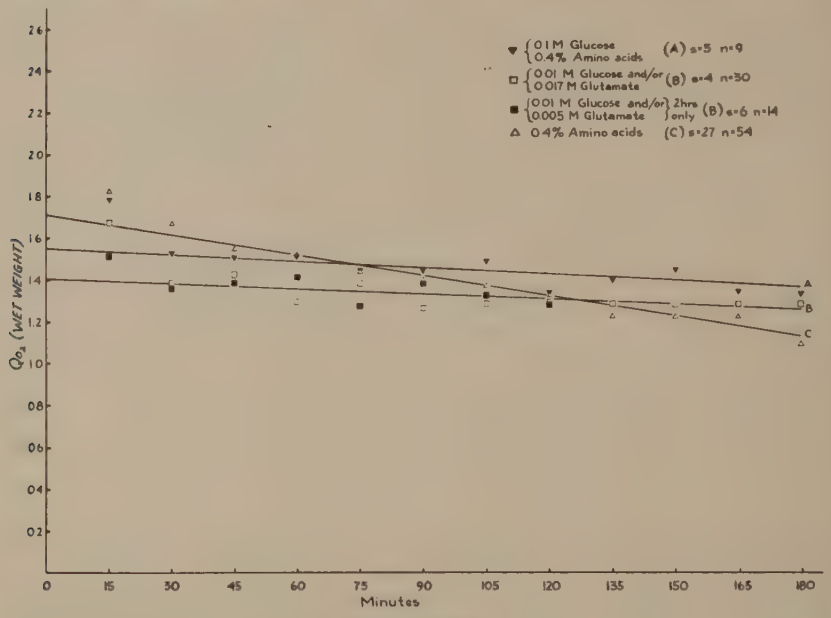

Fig. 3 The Q_{O_2} of human cerebral cortex slices in the presence of mixed amino acids or glutamate with and without added glucose.

those for pyruvate, falling off 23% in three hours. This decrease was prevented by the addition of glucose. Two individual amino acids, DL-alanine and L-glutamic acid, were also used as substrates. Alanine (0.02 M), in accord with the findings for other species (Weil-Malherbe, '36) did not elevate the Q_{O_2} above the endogenous level. Glutamate, known to be important in brain metabolism (Weil-Malherbe, '50) was apparently utilized. It was used in the approximate concentration present in the amino acid mixture (0.005 M) and at

a somewhat greater concentration (0.017 M). Both concentrations produced a Q_{O_2} identical with that found for glucose. Glutamate was also used in combination with 0.01 M glucose since glucose has been shown to influence the accumulation of glutamate in brain cells (Stern et al., '49). However, glucose did not alter the Q_{O_2} found for glutamate alone. In figure 3, the rates for the two glutamate concentrations with and without glucose have been combined for simplicity of presentation. The difference between the metabolism of mixed amino acids and of glutamate shows that although glutamate alone could account for the oxygen uptake produced by the amino acid mixture, either its oxidation is

TABLE 1

Respiratory quotients + S.E. of mean

	NO. VESSELS		R.Q.		Q_{O_2}	
	1st hr.	3rd hr.	1st hr.	3rd hr.	1st hr.	3rd hr.
Endogenous	10	11	0.95 ± 0.02	1.12 ± 0.04	1.19 ± 0.03	0.54 ± 0.04
Glucose	10	11	0.95 ± 0.02	0.96 ± 0.02	1.38 ± 0.04	1.14 ± 0.02
Pyruvate	11	11	1.16 ± 0.02	1.20 ± 0.02	1.71 ± 0.05	1.36 ± 0.07
Succinate	10	7	0.62 ± 0.02	0.33 ± 0.02	2.51 ± 0.11	1.69 ± 0.08
Amino acids	11	11	0.92 ± 0.01	0.92 ± 0.03	1.42 ± 0.06	1.02 ± 0.07

modified by the presence of other amino acids or it is not solely responsible for the oxygen uptake of slices respiring in an amino acid mixture.

While information obtained from respiratory quotient determinations alone is admittedly of limited value, such data were obtained as the initial step in evaluating the nature of the metabolic processes involved in the maintenance of oxygen consumption. Determinations were made for the periods 0–60 and 120–180 minutes without added substrate and with glucose, pyruvate, succinate, and amino acids. The results obtained are presented in table 1. For each time period the Q_{O_2} for each substrate was in the proper order of magnitude, thus serving as a partial check on the accuracy of the respiratory quotient determination.

It is clear that in the absence of added substrate the R.Q. rose between the first and third hours from a value characteristic of hexose oxidation to one compatible with the utilization of oxygen-rich compounds. The quotients for glucose and pyruvate approached the theoretical values expected for their complete oxidation. The amino acid quotients indicate that glutamate is either incompletely oxidized or is not the only component of the mixture which is metabolized since the values are well below the theoretical R.Q. of 1.25 for glutamate oxidation. The low values for succinate are not compatible with its complete oxidation, but rather indicate dehydrogenation to fumarate. During the first hour only pyruvate and succinate yielded respiratory quotients which differed markedly from the endogenous. Third hour values for all substrates except pyruvate deviated markedly from the endogenous, and in all cases may indicate that during the third hour added substrates play a more important part in maintaining respiration. In view of the high endogenous respiratory quotient and the fairly well sustained Q_{O_2} one cannot assume that added substrates are oxidized to the complete exclusion of endogenous compounds even though they are essential to the maintenance of a more constant rate of oxygen uptake.

Further attempts to characterize the oxygen consuming reactions of human cerebral cortex slices have involved the use of inhibitors. Although it is probable that no inhibitor is absolutely specific for a single reaction, some information may be obtained by examining the effects of inhibitors on the oxidation of different substrates, keeping in mind that reaction which is thought to be most sensitive to the inhibitor applied.

Effect of fluoride. It was felt that fluoride might be used to determine whether or not the oxidation of glucose proceeds through the Embden-Meyerhof chain of reactions under aerobic conditions. Further, since the endogenous respiratory quotients were in the range expected for carbohydrate it seemed advisable to test for the presence of glucolytic reac-

tions in the absence of substrate. Since it has been claimed that fluoride inhibits the cytochromes (Borei, '45) as well as enolase, a concentration was determined by experiment which inhibited the oxidation of glucose but not that of pyruvate. Figure 4 shows that 0.015 M sodium fluoride added at 90 minutes significantly inhibited oxygen uptake when the substrate was glucose but had only a minimal effect on pyruvate oxidation and on the endogenous Q_{O_2}. The minimal effect on

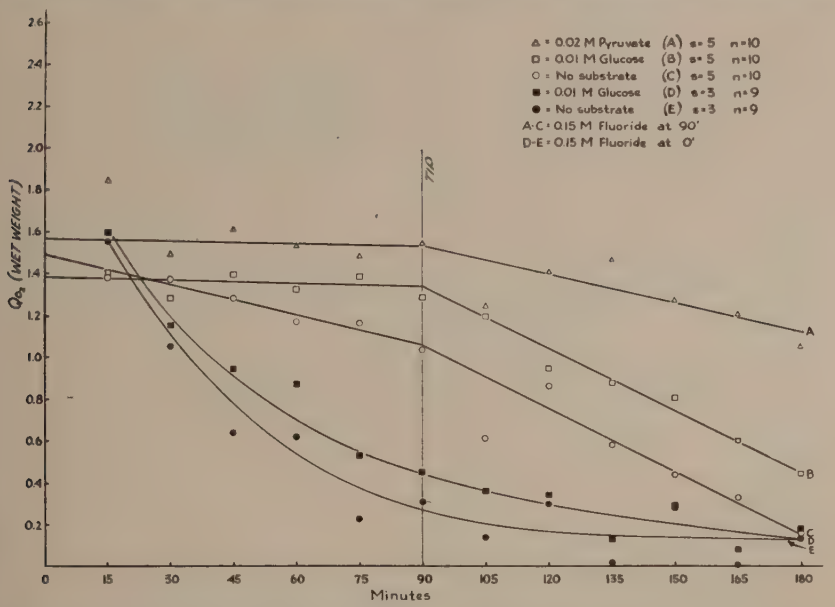

Fig. 4 The effect of 0.015 M sodium fluoride added at zero time and at 90 minutes on the oxygen uptake of human cerebral cortex slices.

pyruvate oxidation connotes lack of action on the cytochromes and suggests that under these conditions fluoride is acting on a glucolytic enzyme, presumably enolase. The fact that glucose oxidation was not inhibited to values below the control endogenous level suggests that the lack of effect of fluoride on the endogenous respiration may be explained on the basis that enolase quite possibly is not the rate determining factor in late endogenous respiration. However, when fluoride was added immediately after the initial

reading, the endogenous Q_{O_2} and the Q_{O_2} in the presence of glucose were inhibited equally, indicating definitely that fluoride sensitive enzymes are concerned with early endogenous respiration. It may be noted that fluoride inhibition develops slowly but leaves practically no residual respiration after equilibrium is attained.

Effect of cyanide. The well known inhibitory effects of cyanide on heavy metal catalysis and the importance of cyanide

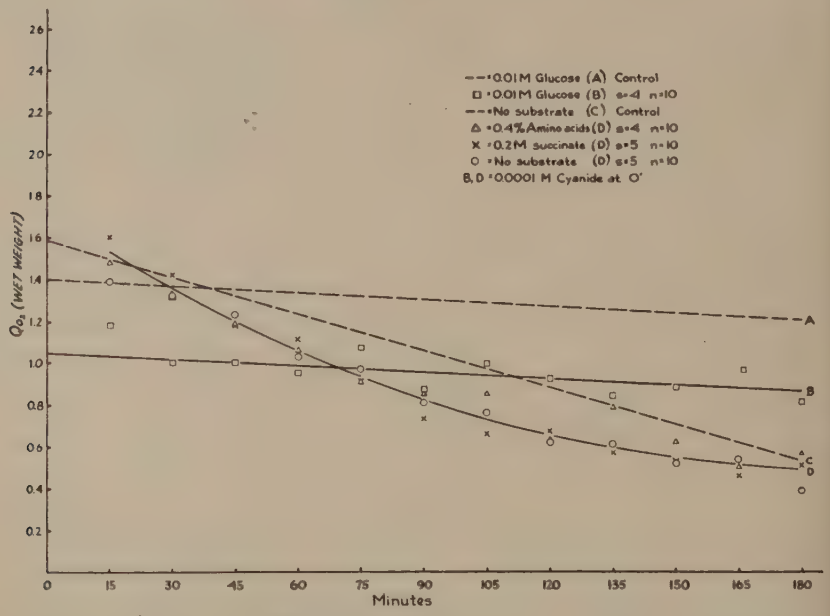

Fig. 5 The effect of 0.001 M cyanide added at zero time on the oxygen uptake of human cerebral cortex slices.

sensitive systems in oxidative processes prompted the study of this agent. The respiration of endogenous origin and with added glucose, succinate, or amino acids was tested for sensitivity to cyanide. Preliminary experiments indicated that 0.0046 M cyanide was a prompt and powerful inhibitor in all cases but when the concentration was reduced to 0.0001 M a differential sensitivity appeared as shown in figure 5. The rate of glucose oxidation was immediately reduced by approximately 30% but continued constant for the remainder of

the three hour run. The endogenous respiration was only slightly inhibited and the higher Q_{O_2} for succinate and amino acids dropped to precisely the same level as the endogenous. The findings suggest that heavy metal catalysts are essential for endogenous respiration as well as for the oxidation of the three substrates tested. However, the endogenous Q_{O_2} is relatively insensitive to cyanide. It is possible that succinate and amino acid oxidation were completely inhibited by the lower

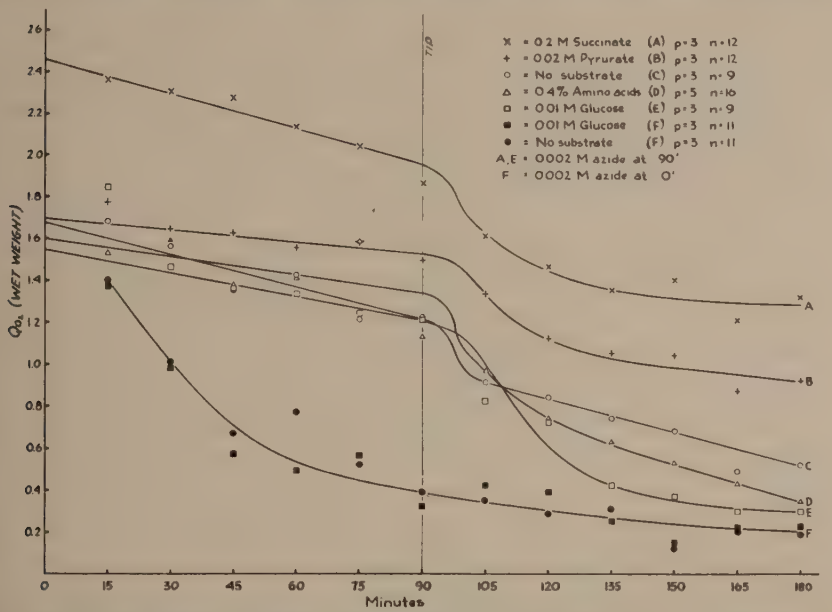

Fig. 6 The effect of 0.002 M sodium azide at zero time and at 90 minutes on the oxygen uptake of human cerebral cortex.

concentration of cyanide, the residual oxygen uptake being entirely of endogenous origin.

Effect of azide. Azide has been shown to inhibit heme containing enzymes such as cytochrome oxidase (Stannard and Horecker, '48) and it interferes with phosphorylation in various systems (Spiegelman et al., '48; Loomis and Lipman, '49). It seemed desirable to compare this agent with cyanide since differences in action have been reported (Stotz, '42; Goddard, '45). Figure 6 portrays the effect of 0.002 M sodium azide

added at 90 minutes on the endogenous respiration and on the oxidation of glucose, pyruvate, amino acids and succinate. Glucose and amino acid oxidation were strongly inhibited, pyruvate oxidation was less affected, inhibition of succinate oxidation was minimal and the endogenous respiration was practically azide-insensitive. However, figure 6 also shows that when azide was added at zero time the inhibition of endogenous respiration was equivalent to that when the substrate was glucose. Comparison with the findings for cyanide shows that oxygen uptake was inhibited by both agents when the substrate was glucose or amino acids but azide contrary to cyanide inhibited to values below the endogenous level. In addition, the endogenous respiration was relatively insensitive to cyanide and to azide added at 90 minutes. The most striking difference in action was on succinate oxidation which was inhibited by cyanide but not by azide. If human brain slices oxidize succinate via the conventional succinoxidase system the latter finding may mean that the principal action of 0.002 M azide in this preparation is on oxidative systems other than those embracing heme containing enzymes.

Effect of malonate. Malonate, considered by Krebs to be practically a specific inhibitor for succinic dehydrogenase (Krebs, '43), was added to slices respiring in the absence of substrate and in 0.2 M succinate. As shown in figure 7 when 0.005 M malonate was added at 90 minutes the oxidation of succinate was inhibited to the endogenous level and the endogenous Q_{O_2} was unaffected. However, when malonate was added at zero time the endogenous Q_{O_2} was definitely inhibited. At 180 minutes the inhibition approximated that attained when malonate was added to succinate at 90 minutes. It appears that succinic dehydrogenase is a factor in the maintenance of the initial endogenous Q_{O_2} but its role is minimized after 90 minutes. It is tempting to assume that succinate oxidation was completely suppressed leaving only the residual endogenous respiration but this assumption must await analytical proof that succinate ceases to disappear after the addition of malonate.

Effect of dibucaine. Of a series of local anesthetics tested by Watts ('49) for their ability to inhibit the respiration of rat brain homogenates, dibucaine was found to be the most potent. Watts reported that these agents block oxidative reactions at the cytochrome c-cytochrome oxidase level and local anesthetics have been reported to inhibit other enzymes (Philpot, '40). The effects of 0.002 M dibucaine added at 90 minutes were determined on the endogenous Q_{O_2} and

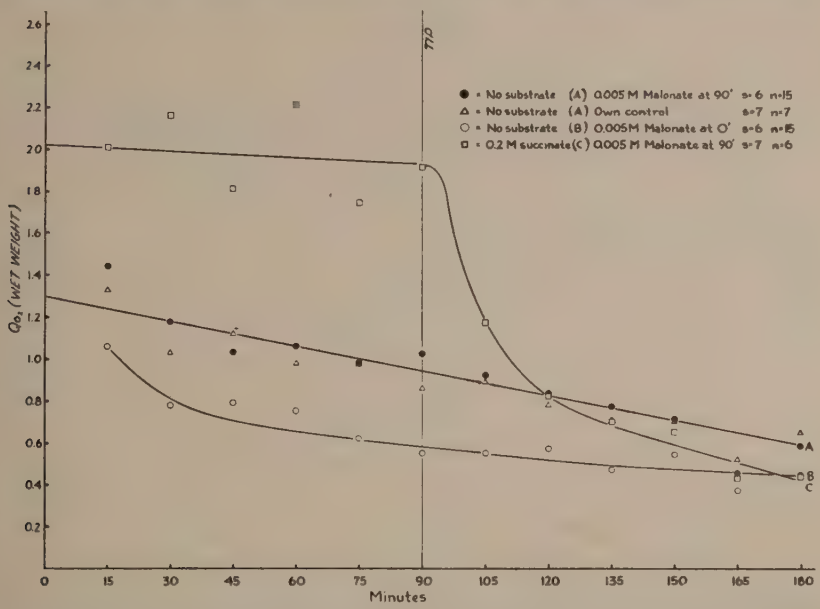

Fig. 7 The effect of 0.005 M malonate added at zero time and at 90 minutes on the oxygen uptake of human cerebral cortex slices.

on the oxidation of glucose and amino acids as shown in figure 8. It is obvious that amino acid oxidation and the endogenous respiration were markedly inhibited within 30 minutes and almost completely inhibited within 90 minutes while inhibition of glucose oxidation progressed more slowly and was not as complete even at 90 minutes. The relative sensitivity of amino acid oxidation to dibucaine as compared to glucose oxidation suggests that it does selectively inhibit the former process and intimates that amino acid or other

amine catabolism may be a factor in the maintenance of the endogenous Q_{O_2}.

Effect of pentobarbital. Central nervous system depressants such as the barbiturates are thought to inhibit biological oxidation at a point between the flavoproteins and the cytochromes (Michaelis and Quastel, '41; Greig, '46). Since extensive studies have been carried out on the brains of other species, the effect of sodium pentobarbital was deter-

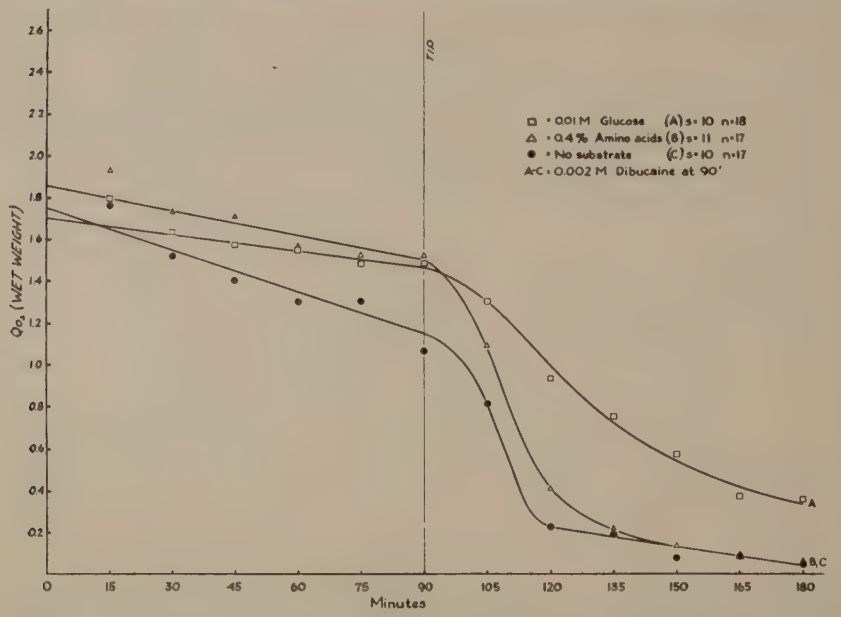

Fig. 8 The effects of 0.002 M dibucaine added at 90 minutes on the oxygen uptake of human cerebral cortex slices.

mined on the endogenous Q_{O_2} and on the oxidation of glucose. The concentration used (0.002 M) was slightly lower than that found by Fuhrman and Field ('43) to produce maximal inhibition of rat cerebral cortex slice respiration. Figure 9 shows that 0.002 M pentobarbital added at 90 minutes inhibited glucose oxidation more than the endogenous Q_{O_2}, the effect reaching a maximum within 60 minutes after drug addition. Since the pentobarbital-stable respiration was higher in the absence of added substrate than when glucose

was present, it is unlikely that the reactions responsible for oxygen uptake are identical in the two cases. A concentration action curve for pentobarbital was not determined, but judging from the degree of inhibition produced by the concentration used, human cerebral cortex slices are slightly more sensitive to pentobarbital than rat brain slices.

Effect of the absence of Ca^{++}. Media containing no calcium have often been used for the study of tissue metabolism

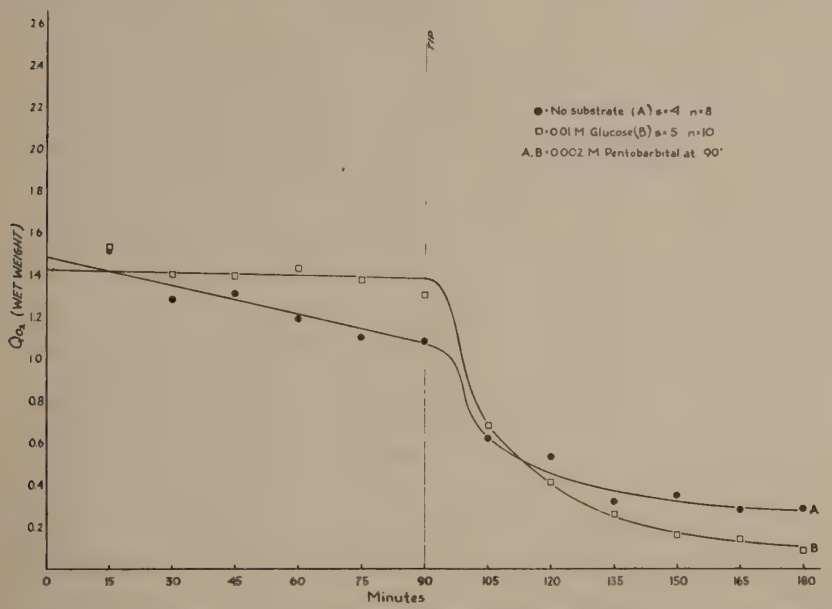

Fig. 9 The effects of 0.002 M sodium pentobarbital added at 90 minutes on the oxygen uptake of human cerebral cortex slices.

because of the higher Q_{O_2} obtained thereby (Dickens and Greville, '35) and because of the variable precipitation of the ion in the presence of phosphate (Elliott, '48). In order to have a comparison of the metabolism in the presence and absence of Ca (Elliott, '48; Elliott and Penfield, '48), we have made a study of the metabolism of human brain slices in calcium free Ringer's with the results depicted in figure 10. Apparently, when calcium was omitted from the media the glucose Q_{O_2} was increased more than 10%. There was

no effect when the substrate was mixed amino acids but when glucose was also present the Q_{O_2} was increased above the level found for glucose alone and remained constant for three hours. On the other hand, the endogenous Q_{O_2} was slightly depressed; indicating that in this case calcium is involved in the maintenance of oxygen uptake.

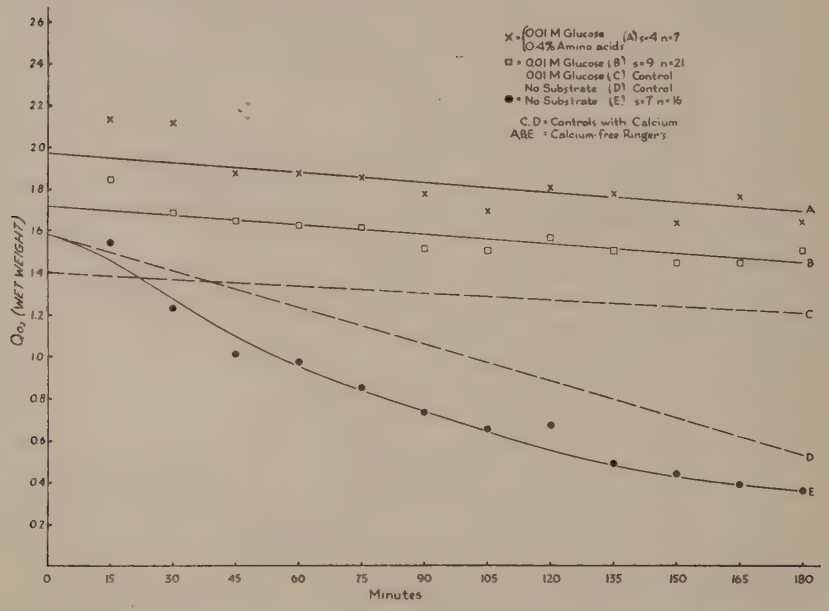

Fig. 10 The oxygen uptake of human cerebral cortex slices in calcium free Ringer's.

DISCUSSION

If gucose is the usual fuel of the brain, then the Q_{O_2} in glucose-Ringer's should be the most suitable for comparison with *in vivo* metabolism studies. Under our conditions, the Q_{O_2} for cerebral cortex slices respiring in 0.01 M glucose was 1.4 when extrapolated to zero time. Since in unpublished experiments the Q_{O_2} of human cerebellum and cerebral white matter were found to be 1.2 and 0.3 respectively, it is possible to make a rough estimate of the oxygen uptake of the whole brain as determined *in vitro*. Assuming a brain weight of 1300 gm subdivided according to Count ('47) and Jakob ('27),

the contributions of the various parts to a theoretical oxygen consumption of whole brain are as follows: 632 gm of cortical gray matter, 14.5 cm^3 per minute; 499 gm of cortical white matter, 2.5 cm^3 per minute; and 143 gm of cerebellum, 2.9 cm^3 per minute. Assigning to 26 gm of pons and medulla a rate one-half that of cerebral cortex makes their contribution 0.3 cm^3 per minute. The total, 20.2 cm^3 per minute amounts to 74% of the *in vivo* value of 27.3 cm^3 per minute determined by Kety ('40) and coworkers for subjects under thiopental anesthesia, in whom functional activity of the brain is probably at a very low level. Considering the vast difference between *in vitro* and *in vivo* conditions this comparison shows that values obtained with slices are not completely incompatible with intact brain studies.

In accord with the findings for excised brain tissue from other species, it has been determined that the Q_{O_2} of human cerebral cortex slices can be maintained above the endogenous level by the addition to the suspending medium of certain hexoses, three carbon intermediates of glucose metabolism, and other compounds capable of oxidation through the tricarboxylic acid cycle. The appreciable endogenous respiration, plus the fact that except in the presence of pyruvate respiratory quotients did not exactly equal theoretical values for the substrates used makes it unlikely that any one substrate serves as the sole energy source in brain slices.

Although various substrates will maintain the Q_{O_2} of human brain slices, of those used in these studies only glucose (Himwich et al., '41) and glutamate (Mayer-Gross and Walker, '49) have been found capable of reversing hypoglycemic coma in man. The possibility that amino acids may serve as a foodstuff for the intact brain lends special significance to our findings with glutamate and mixed amino acids. Unlike rat brain, human brain slices can derive oxidative energy from amino acids in the absence of glucose, however, the amino acid Q_{O_2} is more constant when glucose is present. The use of glutamate alone which could not maintain the Q_{O_2} of rat or guinea pig brain slices (Weil-Malherbe, '36) also results

in a constant Q_{O_2} which is not influenced by glucose or a three fold variation in glutamate concentration. Thus it appears that amino acids are utilized more efficiently by human brain slices than by those of other species and may explain in part the *in vivo* findings with glutamate.

The effects of inhibitors provide some information as to the nature of the metabolic processes operating in human brain slices. The marked inhibitory action of 0.00046 M cyanide on respiration, endogenous as well as with added substrates strongly indicates that heme containing enzymes such as the cytochromes are of general importance, although quantitative differences exist as shown by the effects when the concentration was lowered to 0.0001 M. The actions of azide do not exactly parallel those of cyanide and it is possible that this inhibitor does not specifically inhibit iron containing enzymes under the conditions of our experiments. Fluoride and malonate appear to exhibit the actions ascribed to them by previous workers. The fact that these agents, and azide as well, are more potent inhibitors of endogenous respiration when added at zero time suggests an alteration of endogenous metabolic processes with time. Supportive evidence for this is provided by the respiratory quotients obtained in the absence of added substrate. A possible link between endogenous metabolism and amino acid oxidation is found in the ability of dibucaine to inhibit markedly the oxygen uptake of slices respiring in unfortified or in amino acid Ringer's while having less effect when the substrate was glucose. However, until the effects of this agent on tissue metabolism have been more thoroughly investigated, conclusions regarding its actions must necessarily be tentative.

Finally the respiratory rates obtained in calcium-free media are of some interest. With glucose as the substrate the 30 minutes' Q_{O_2} was 1.76, very close to 1.80 the value predicted for man by K. A. C. Elliott ('48). The increase in Q_{O_2} over that in ordinary Ringer's resembles that produced by raising the potassium concentration (Lipsett and Crescitelli, '50) and may be considered a manifestation of the

well known biological calcium-potassium antagonism. Stimulation was evident only when glucose was present in the medium; a result which together with the existence of the calcium-potassium antagonism quite likely reflects the findings of Boyer et al. (Boyer et al., '42, '43) that potassium catalyzes transphosphorylation in the Embden-Meyerhof chain of glucolytic reactions.

SUMMARY

The oxygen uptake of human cerebral cortex slices under various conditions has been studied by means of conventional manometric techniques. In unfortified Ringer's an appreciable Q_{O_2} was found which diminished lineally with time. The addition of glucose, fructose, galactose, pyruvate or lactate resulted in a Q_{O_2} which remained essentially constant for three hours. With added succinate, the Q_{O_2} paralleled the endogenous rate and within limits was dependent on succinate concentration. Mixed amino acids and glutamic acid, but not alanine, also supported respiration in the absence as well as presence of glucose. Respiratory quotient determinations in unfortified Ringer's suggested hexose oxidation followed by utilization of oxygen rich compounds. With added glucose, pyruvate or succinate only the pyruvate R.Q. approached the theoretical value for complete oxidation. The amino acid R.Q. was compatible with the utilization of more than one component of the mixture. Inhibition of oxygen uptake by fluoride, cyanide, azide, malonate, dibucaine, and pentobarbital was determined in the presence of appropriate substrates and in unfortified Ringer's. Inhibition of the endogenous respiration by fluoride, azide, and malonate was more effective when these agents were added at zero time than at 90 minutes. Oxygen uptake was increased in calcium free Ringer's with added glucose and amino acids but endogenous respiration was slightly depressed.

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Boldrey, J. E. Adams, and the resident staff of the Division of Neurological Surgery in obtaining the biopsy material. Thanks are also due Dr. J. J. Eiler for a critical review of the manuscript.

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FRACTIONATION OF EMBRYO EXTRACT BY ULTRACENTRIFUGATION

I. ANALYSIS OF FRACTIONS[1, 2]

JEROME J. WOLKEN[3, 4]

FIVE FIGURES

INTRODUCTION

Since the early work of Carrel ('21) and his collaborators, who demonstrated the growth-promoting activity of chicken embryo extract, attempts have been made to further isolate and characterize the components responsible for this growth stimulation (Fischer, '46). Later, Claude ('38) isolated by differential centrifugation a fraction from normal embryonic tissue rich in nucleic acid, and Tennent, Leibow, and Stern ('41) showed that a similar fraction isolated from chicken embryo extract accelerated the growth of mouse heart fibroblasts in tissue culture. But Claude ('49), Fischer ('46), Brachet ('43, '45) and others showed that this growth-stimulating effect was present in the supernatant fluid as well.

Previous growth studies by Wolken ('48, '50) on the incorporation of P^{32} into the developing embryo suggested a tracer method as an aid in fractionation and analysis. The experimental procedures which follow are an attempt to isolate by differential centrifugation tagged fractions which may be tested for their growth-stimulating effect, and to further characterize them as to their chemical nature and physical properties, such as sedimentation constant, size, shape, and homogeneity.

[1] Presented at the 119th meeting of the American Chemical Society, Division of Biological Chemistry, Boston, April, 1951.

[2] Publication No. 14 of the Department of Biophysics, University of Pittsburgh, Pittsburgh, Pennsylvania.

[3] American Cancer Society Fellow on the recommendation of the Committee on Growth of the National Research Council.

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EXPERIMENTAL METHODS AND RESULTS

Fractionation of tissue and analysis

Five-tenths μc of P^{32} as sodium hydrogen phosphate (pH 6.8) was injected per fertile egg just below the air space and the eggs incubated at 37°C. After 8 days of incubation, the eggs were broken and only the viable embryos removed. The pooled embryos were then homogenized with an equal weight of physiological saline (0.9% NaCl) in a Potter-Elvehjem homogenizer. All operations were carried out in an ice bath under sterile conditions. Large particles were removed by low speed centrifugation and the resulting extract stored at 5°C. for further treatment as shown schematically in figure 1.

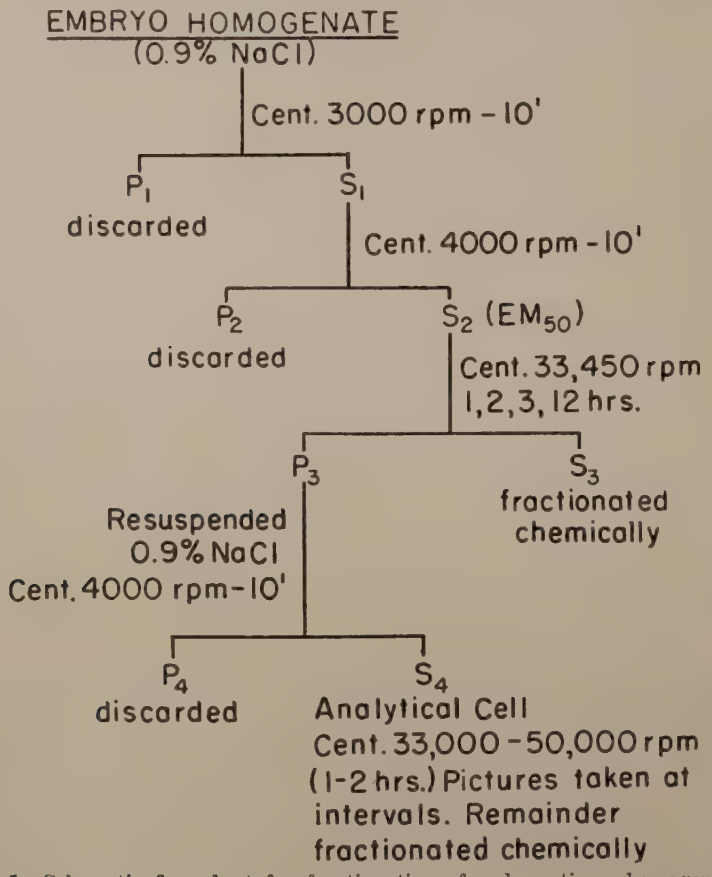

Fig. 1 Schematic flow sheet for fractionation of embryo tissue homogenate.

All high speed centrifugation was carried out under refrigeration in the Spinco model E ultracentrifuge. All fractions indicated as discarded were chemically analyzed for phosphorus compounds but not tested for any biological activity.

The per cent of incorporation of P^{32} was determined for the embryo before and after fractionation. The pellet, supernatant fluid, acetone precipitate, and dialyzed material obtained from the embryo homogenate were further fractionated chemically into acid-soluble, phospholipid, nucleic acid, and phosphoprotein.

The radioactivity was measured by removing an aliquot, diluting to a known volume, and then removing an additional

TABLE 1

Fractions in counts/minute

	S_2	S_3	S_4
Total before fractionation	97,635		9,912
Low speed pellet	65,800		6,580
Supernatant fractions	30,330	19,950	3,224
Acid sol. P	18,018	16,263	624
Phospholipid P	4,012	2,470	466
Nucleic acid P	7,770	936	1,648
Phosphoprotein P	1,680	780	168
Recovered	31,480	20,449	2,906

aliquot which was spread on aluminum pans and evaporated under an infra-red lamp. All samples were counted using a thin-window geiger counter. The chemical fractionation of the phophorus compounds and their analyses were those of Schneider ('46) and the method of Schmidt and Thannhauser was used for the RNAP and DNAP ('45). As a further precaution, preliminary analyses were run to detect the possible contamination of the samples by radioactive phosphate. In most cases it was found to be no greater than 1%. The method for the acetone extraction was that of Margoliasch ('50).

The results of the fractionation and analyses are summarized in tables 1, 2, and 3. It was found that an average

of 10% of the injected P^{32} is incorporated into the developing embryo by the 8th day of incubation.

Table 1 is a breakdown in terms of counts per minute of the various fractions and degree of recovery possible. It will also be noted that two-thirds of the radioactive phosphorus in the original homogenate and high speed fractions are removed by low speed clarification. The low speed centrifugation also removes all of the desoxyribose nucleic acid phosphorus (DNAP) of the original homogenate as can be detected by our techniques.

TABLE 2

Average % P^{32} activity

FRACTION	TOTAL	ACID SOLUBLE P	PHOSPHO-LIPID P	NUCLEIC ACID P	PHOSPHO-PROTEIN P
Embryo extract S_2	100	61	10	21	8
Supernatant S_3	70	83	8	6	3
Supernatant S_4	30	16	17	58	9
Acetone ppct. from S_3	20	80	3	9	8
Acetone ppct, from S_2	89	88	3	4	5
Dial. S_2	65	7	30	8	55

Table 2 is an average of the per cent activity of P^{32} retained by the various fractions in terms of acid soluble, phospholipid, nucleic acid, and phosphoprotein phosphorus. It will be noted that an average of 30% P^{32} is retained in the fraction S_4, and 58% of that is incorporated into the nucleic acid fraction; whereas in the supernatant fluid S_3, 83% of the P^{32} is in the acid soluble fraction and only 6% in the nucleic acid fraction. After 12 hours of ultracentrifugation, positive tests for nucleic acid P were still obtained for the supernatant fluid. This experimental information may lend some support to the idea that a nucleic acid complex of the ribose type is essential for growth stimulation and explain in part why others have found that both the pellets and supernatant fluids are biologically active.

Table 3 is similar to table 2 except that here can be noted the amount of ribose nucleic acid phosphorus (RNAP) recovered in terms of counts per minute and per cent activity. The specific activity or rate of turnover of radioactive P^{32} to normal P^{31} for all fractions is given.

TABLE 3

	S_2			S_3			S_4		
	c/m	% P^{32}	Spec. act.	c/m	% P^{32}	Spec. act.	c/m	% P^{32}	Spec. act.
Acid soluble P	2574	57.2	10.7	2502	79.8	11.2	310	20.3	1.8
Phospholipid P	576	12.8	1.9	368	11.7	1.3	230	15.0	1.4
Nucleic acid P	1110	24.7	6.8	144	4.6	1.3	905	59.2	6.9
RNA P	960	21.3	6.1	140	4.5	1.3	838	54.8	6.4
Phosphoprotein P	240	5.3	.8	120	3.8	.9	84	5.5	.9
Total (— RNA P)	4500			3134			1529		

Spec. act. (specific activity) — counts per minute per microgram of total phosphorus.

Nitrogen-phosphorus

The nitrogen determinations were made using the micro-Kjeldahl method. The phosphorus analysis was determined with the Beckman spectrophotometer according to the procedure of Kitson and Mellon ('44). The high speed fraction S_4 was found to contain 10–12% nitrogen, whereas the P/N for the nucleic acid fraction of S_4 was from 20–33%.

Analytical ultracentrifugation

The high speed fractions S_4 obtained from each fractionation were subjected to analytical ultracentrifugation in order to measure their degree of homogeneity and apparent sedimentation constant. Figure 2 is a typical example. It will be noted that at least two peaks and possibly a third can be detected, indicating that there are two or three particles of different sizes present in high concentration. Attempts to separate these out by use of the separation cell were unsuccessful. The average uncorrected sedimentation constants were found to be 10 S, 25 S, and 50 S (in Svedberg units).

Fig. 2 Analytical ultracentrifuge photographs of S_4. Analyzed at concentrations of 15–20 mg per milliliter in .9% NaCl.

Absorption spectra

The Beckman spectrophotometer was used to measure the absorption spectra for the high speed fraction S_4, supernatant fluid S_3, acetone precipitate of S_3, and acetone precipitate of the embryonic fluid S_2.

It was found that the S_4, acetone precipitate of S_3, and the acetone precipitate of the embryonic fluid strongly absorb

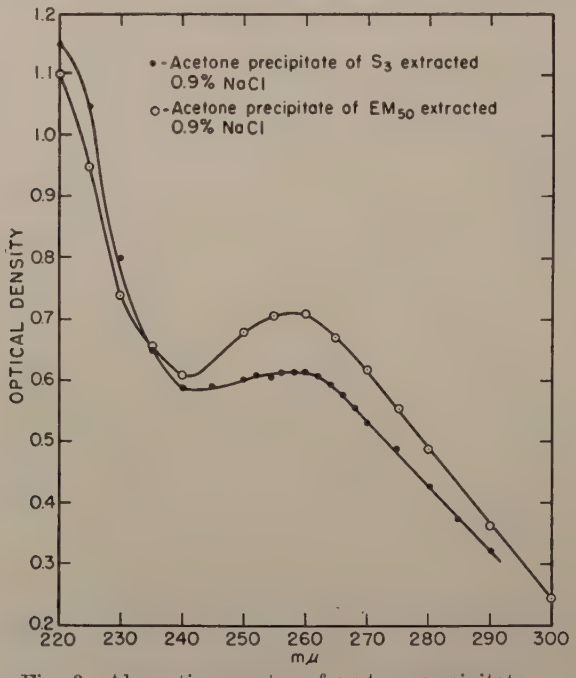

Fig. 3 Absorption spectra of acetone precipitates.

at 260 mμ (fig. 3), whereas the supernatant fluid S_3 strongly absorbs at 410 mμ (fig. 4). If HCL is added to fraction S_3, the absorption band shifts to 380 mμ. This shift may be due to the nucleic acid still present in the supernatant fluid which is not sedimented out. The 410 mμ is the Soret band perhaps for coproporphyrin which is at its highest concentration in the 9-day-old chicken embryo.

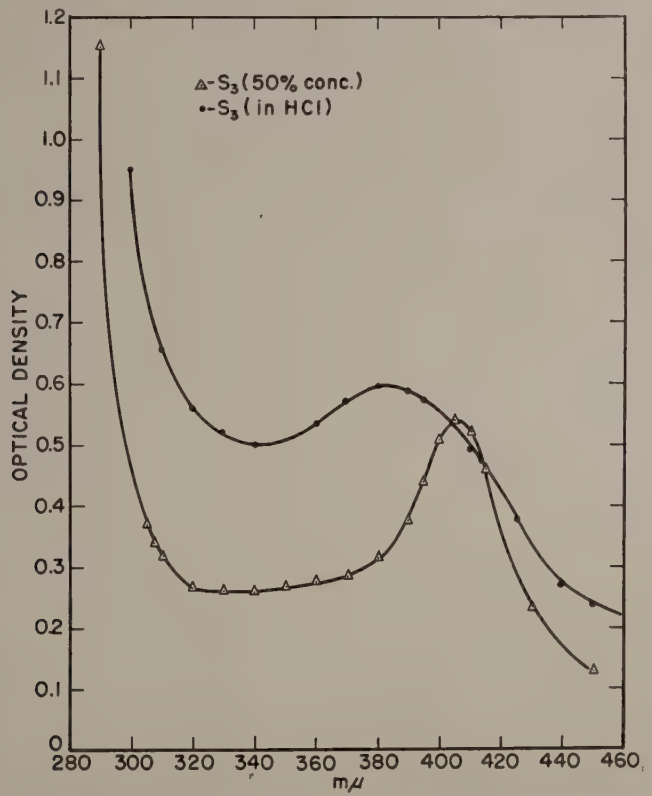

Fig. 4 Absorption spectra of supernatant fluid S_3.

Electron microscopy

Samples of fraction S_4 were diluted with distilled water and dried in vacuum. Some were fixed with 4% formaldehyde and dialyzed against distilled water at 5°C. and all were shadowed with uranium. All the electron micrographs show

a uniformity of size. Figure 5 is a typical example showing distinct isolated particles. The particles appear to be spherical and the average observed size-range was found to be between 20 and 50 mμ.

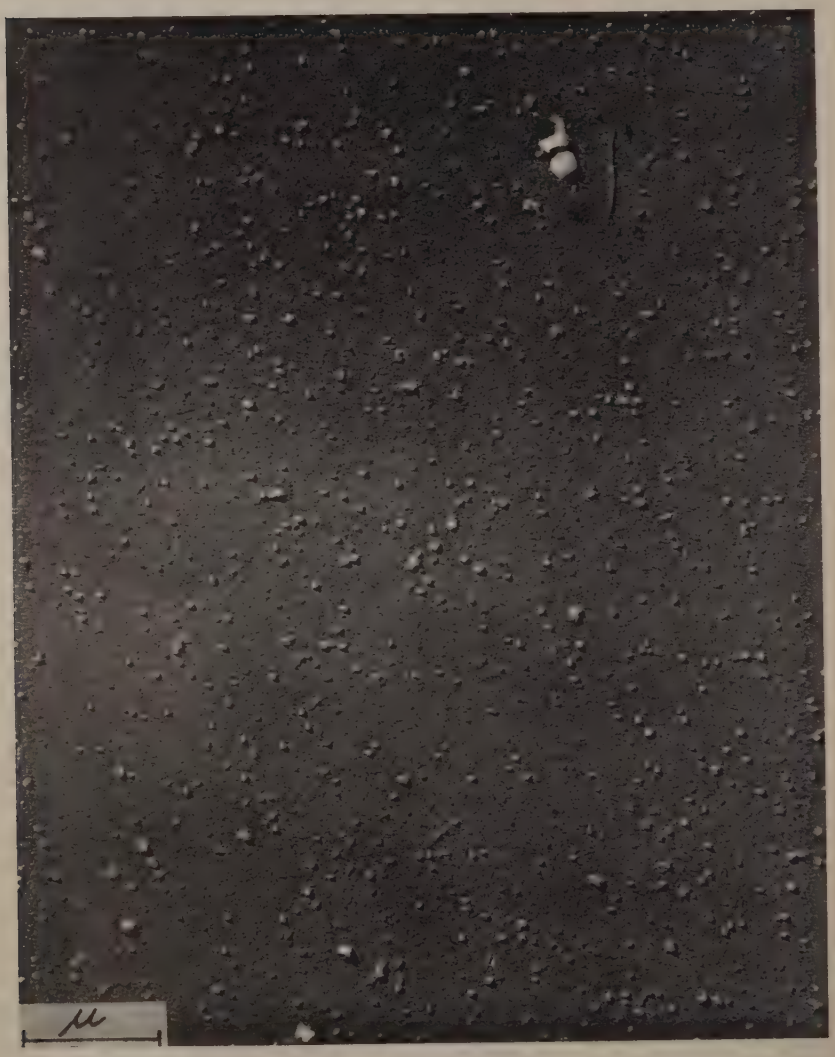

Fig. 5 Electron micrograph of S_4. Fixed with 4% formaldehyde, dialyzed, and shadowed with uranium. × 30,000.

Biological tests

Samples of the S_3, S_4, acetone precipitate of S_3, and acetone precipitate of S_2 were added, in a typical tissue culture experiment, to a Tyrode's solution in which chicken heart fragments were growing.

The measure of areal spreading of the excised heart fragments after the addition of the test fractions and 48 hours' incubation at 37°C., was taken as a qualitative indication of growth. Since comparable areal spreading was obtained from all cultures, no quantitative differences can be reported. Neither did fractions S_3 and S_4 agglutinate chicken red blood cells. Additional attempts at quantitative tests for biological activity are now in progress.

DISCUSSION

The concentrated supernatant fluid S_3 has previously not been of much interest, although it has been shown to have growth-stimulating activity. It was found to contain RNAP which cannot be completely sedimented even at 50,000 r.p.m. and 12 hours of centrifugation, and it is mostly low molecular weight acid-soluble compounds. It is also rich in hemin-like material, probably coproporphyrin.

On the other hand, the fraction S_4 is of considerable interest. It is a heterogeneous preparation of two or three homogeneous spherical particles in high concentration, with an average diameter of 20–50 mμ. It was found that a high ratio of radioactive P^{32} to normal P was incorporated in this fraction with an average of 50–60% in the RNA, 15–20% in the phospholipid, 5–10% in the phosphoprotein, and 15–20% in the acid soluble.

These are not dissimilar to virus-like particles in size, shape, and chemical composition. The particles also appear to be visually identical with electron micrographs of human cancerous milk and cancerous breast tissue recently published by Passey, Astbury et al. ('51).

These particles are not identical with any morphological cytoplasmic particulate known at present, but could be considered as the ultra-microsome fraction.

Additional studies are being carried on regarding the nature and role of similar particles found in the yolk of hen's egg and their relationship to those found in the embryo.

SUMMARY

Investigation has been carried out on the *in vivo* incorporation of radioactive P^{32} into the chicken embryo and its subsequent fractionation. Methods for isolating these fractions from the tissue homogenates by differential centrifugation, acetone extraction, and dialysis are described and their properties further characterized by chemical analyses, spectroscopy, analytical ultracentrifugation, electron microscopy, and biological activity.

Ultracentrifugation of the embryo extract results in removal of 87% of the total solids, which contain 30% of the total radioactive phosphorus. The pellet S_4 contains 50–60% P^{32} in the ribose nucleic acid fraction, 10–12% of the total nitrogen, has apparent sedimentation constants S10 to S50, and an average size of 30 mμ with many smaller and larger particles present. The supernatant fluid S_3 contains 70% of the radioactive phosphorus of which 80–85% P^{32} is in the acid-soluble fraction. It is also rich in hemin-like materials which absorb at 410 mμ. The acetone precipitate is similar to the supernatant fluid S_3.

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INJURY TO LIVING CELLS IN STANDING SOUND WAVES [1, 2]

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EIGHT FIGURES

INTRODUCTION

When intense high frequency sound or ultrasound passes through a liquid medium, a number of distinctive phenomena may be observed (Wood and Loomis, '27). Among these are the generation of heat, the appearance of vapor or gas-filled cavities or bubbles, circulation of the liquid, and the appearance of oxidation products in water. Living cells or tissues placed in the path of the sound may be rapidly injured or killed, but the mechanisms by which the damage is produced are not always clear.

The heat generated by sound absorption may be sufficient to produce thermal coagulation. However, injury may also occur when heating is absent, and in such cases, the type of injury produced differs from thermal coagulation, resembling rather that produced by blows with solid objects. This mechanical injury is characterized by swelling of the nucleus and cytoplasm followed successively by displacement of chloroplasts, when present, dissolution of the nucleus, vacuoles and other cell bodies, coagulation and eventual mixing and dispersion of the cell contents (Lepeschkin, '27; Lepeschkin and Goldman, '52). It has been attributed to the

[1] The opinions or assertions contained herein are the private ones of the writers and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large.

[2] Presented in part at meeting of Acoustical Society of America, State College, Pennsylvania, June, 1950.

effects of cavitation [3] since it is abolished or greatly reduced by degassing the liquid medium or by increasing the hydrostatic pressure, both of which procedures markedly raise the sound level required to produce the cavitation (Johnson, '29; Harvey, '30; Chambers and Gaines, '32). Furthermore, the addition of gelatin to erythrocyte suspensions prevents the formation of bubbles and inhibits hemolysis (Harvey, '30). Careful observation has failed to reveal evidence of intracellular cavitation (Harvey, '30; Harvey and Loomis, '31; Lepeschkin and Goldman, '52). This is plausible in view of the high viscosity of protoplasm and the fact that plant cells may have an internal turgor pressure of several atmospheres. It has been surmised (Rouyer and Grabar, '47) that the mechanical injury is produced primarily by the intense shock waves accompanying the formation and collapse of the cavities (Rayleigh, '17).

If a reflector is placed opposite the sound source it is possible to set up rather well defined standing waves in the medium. There is some evidence of a difference between the effects produced at nodes and at antinodes. Protozoa, being able to swim between the nodes of the stationary wave system set up in the water, escape unharmed (Schmitt, Olson and Johnson, '28). Erythrocytes accumulate at nodes of vibration and do not show any hemolysis (Dognon and Biancani, '37). Under high pressure red blood cells collect at nodes and are not destroyed (Johnson, '29). Perusal of early literature in acoustics (Kundt and Lehmann, 1874; Rayleigh, Theory of Sound), indicates that the terms node and antinode when unqualified refer to the particle motion of the medium. More recently, however, this usage may not have been uniformly followed. In any case, cavitation necessarily occurs at pressure antinodes although the visible bubbles accumulate at pressure nodes (Goldman and Ringo, '49).

[3] Cavitation is not yet a well understood phenomenon. For the purposes of this paper it may be defined as the occurrence of gas or vapor-filled cavities or bubbles at high sound levels in liquids.

The experiments described here are intended as a further study of the damage to living cells produced in standing wave systems where nodal and antinodal effects can be separated.

METHODS AND MATERIALS

Standing sound waves at frequencies of 400, 700 and 1,000 kc. were produced in a cylinder of water having a thin glass bottom and placed over a quartz crystal immersed in an

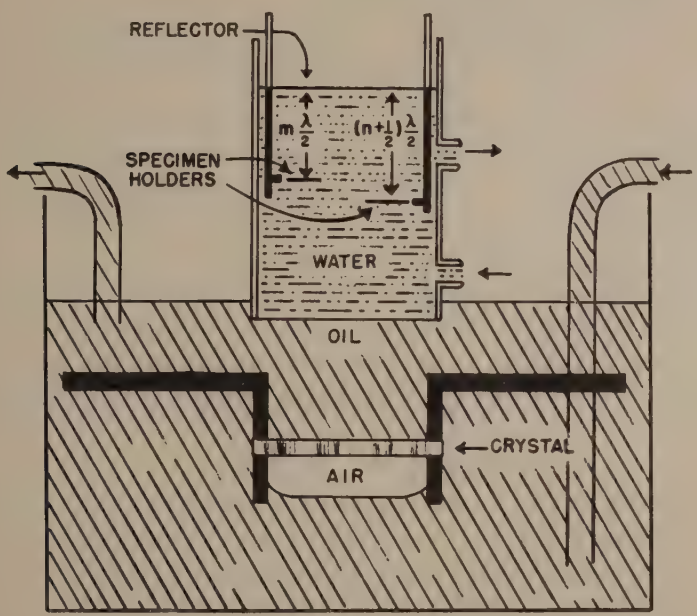

Fig. 1 Schematic diagram of apparatus for exposure of specimens to ultrasound at pressure nodes or antinodes.

oil bath using a Crystal Research Laboratories Ultrasonorator (Lepeschkin and Goldman, '52) (fig. 1). Metal foil or a glass cover slip, thin compared to the wave length of the sound, was placed at the air-water reflecting interface to insure that this surface would be a pressure node and that the surface would remain plane. Care was taken that the significant surfaces were plane parallel and that any parts of the various holders, etc., would provide a minimum of in-

terference with the formation of a plane wave system. The success of these procedures could be roughly judged by the appearance of plane parallel layers of air bubbles at half wave intervals; and the position of a node or antinode could be readily determined either by reference to the bubble layers or by measurement of the distance from the reflector.

The accurate measurement of sound intensities when above the level required to produce cavitation has so far proved impracticable. Accordingly, the DC volt-ampere product in the final amplifier stage of the electrical generator was used as a rough index of the power delivered. This procedure was justifiable only for comparison purposes and then only when the acoustic impedance of the entire crystal and specimen holder system was kept constant as well as the arrangement of all metal objects close to the step-up transformer in the oil-filled jar.

Temperatures were maintained within 5°C. near room temperature by circulating cooled oil through the bath in which the crystal was mounted, and when necessary by circulating cooled water through the cylinder containing the specimens.

The gas content of the water was determined by Winkler's method.

Human erythrocytes, yeast (National Yeast Corporation compressed yeast), leaves of *Elodea canadensis,* an infusorian *(Holophria,* Ehrenberg), an undetermined species of rotifer and two species of Spirogyra were used. One species of Spirogyra had cells 66 μ thick and about 140 μ long, with 4 chloroplasts per cell; the other had cells 126 μ thick, about 300 μ long, and 7 chloroplasts per cell.

Erythrocytes from oxalated human blood were suspended in 0.95% NaCl buffered to pH 7.3 with isotonic phosphate made up with water distilled in glass. Yeast cells were suspended in tap water buffered to pH 7.1 with 0.05% phosphate. Infusoria and rotifers were suspended in tap water. The suspensions were exposed to the ultrasound in a small chamber made of glass cover slips and sealed with cement or wax.

Elodea leaves were fastened to a glass or lucite slide with fine glass hairs cemented to the slide.

Spirogyra filaments, usually 10 to 15 mm long, were inserted in a cage consisting of a pair of fine glass hairs fixed to a cover slip and held down by a few hairs laid across the others and also fastened with cement. The pH of the tap water was adjusted to 7.3 with 0.03% phosphate.

For experiments under reduced pressure the glass cylinder in which the preparations were mounted was connected to an aspirator pump. For high pressure experiments a cylindrical steel cylinder was used which had a bottom of dural the thickness of which was half a wave length.

The effects of the ultrasound were determined by microscopic examination. For Spirogyra the criterion of minimal injury was displacement of chloroplasts. For yeast cells, a mixture of equal volumes of 0.02% Neutral Red and 1% NaCl solution was added to an equal volume of cell suspension; injured and dead cells take the stain readily. If the yeast cells were imbedded in agar, slices were soaked in the solution for half an hour. Hemolysis of erythrocytes was determined with a Klett-Summerson photoelectric colorimeter, or by cell counts if the cells were imbedded in agar.

RESULTS

When Spirogyra filaments were exposed in air saturated water to the standing waves with the filament axes in the direction of the field (vertically), injury was produced at half wave intervals (fig. 2) and occurred primarily at the pressure antinodes (fig. 3). With Elodea, a clear picture was more easily obtained (fig. 4). Long continued exposure at moderate or high intensities produced injury to all cells. Similar results were obtained with suspensions of infusoria, rotifers and erythrocytes. A suspension of infusoria placed at a pressure node showed no injury after two and one-half minutes' exposure (200 volt-amperes, 0.7 mc.), whereas a similar preparation at a nearby pressure antinode showed, for the same exposure time and intensity, severe damage with no

recovery in 24 hours. Exposure of rotifers under the same conditions at the pressure node produced no effect, and exposure at the antinode produced a complete stoppage of movement although no destruction occurred. Exposure of erythrocytes at a pressure node (150 volt-amperes, 0.4 mc.) produced complete hemolysis in 30 to 40 minutes; exposure at an antinode produced complete hemolysis in 4 to 6 minutes.

When the aqueous medium was degassed by boiling and by pre-exposure to intense ultrasound and the organisms then

Fig. 2 Spirogyra filament after exposure to ultrasound at 400 kc. showing coagulated cells at regular intervals.

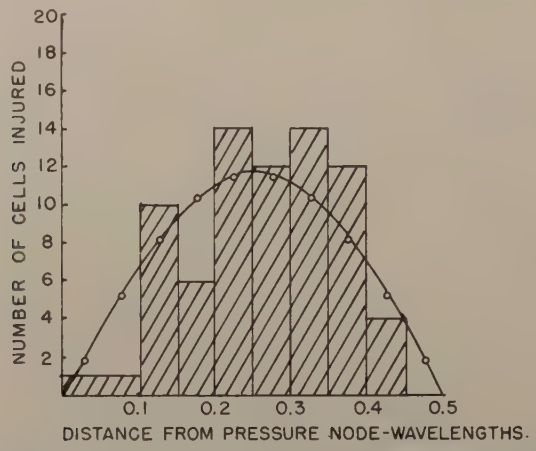

Fig. 3 Cell injury along sound field showing statistical distribution of injured cells. Smooth curve is a sine wave scaled for the same total number of injured cells. Data taken at 400 kc. and 1 mc.

exposed, the injury was much less and appeared first at the pressure nodes. Long filaments of Spirogyra were cut into several pieces, and the pieces exposed horizontally with their axes perpendicular to the direction of sound propagation. Two pieces were exposed simultaneously, one at a pressure node and one at a nearby antinode. After a few minutes the number of injured and dead cells was counted. A series of such experiments on pieces from the same long filament, exposed to different intensities at different gas concentrations, showed a decided effect of both intensity and gas content.

Fig 4 Left: Elodea leaf after exposure to ultrasound showing damaged areas at regular intervals. Right: Elodea leaf in sound field showing relation of bubble striations (pressure nodes) to areas of damage (400 kc.).

In gassy water, no detectable injury occurred below a critical power input (probably the cavitation level) above which injury increased rapidly both at nodes and antinodes. In water which was only 5% saturated with air, injury was first seen at a higher sound level, appeared first at a pressure node, and increased slowly with increasing power input. In 2% agar, however, no injury at all occurred at the pressure antinode up to the maximum power available while injury at the pressure node increased steadily from a low input power level (fig. 5). Agar gel has a powerful inhibiting effect on the formation of cavities although its acoustical properties (sound velocity, absorption, density) are very

close indeed to those of water. A simple demonstration of the effects of using an agar medium was carried out by casting a saline agar erythrocyte suspension about half an inch thick in the bottom of the glass cylinder, adding a few inches of the same suspension without agar above and then exposing to ultrasound. An exposure sufficient to hemolyze the aqueous suspension had very little effect on the agar imbedded cells. Further comparative data on the injury at nodes and antinodes in tap water and in agar gel confirmed these effects. Paired halves of Spirogyra filaments were exposed

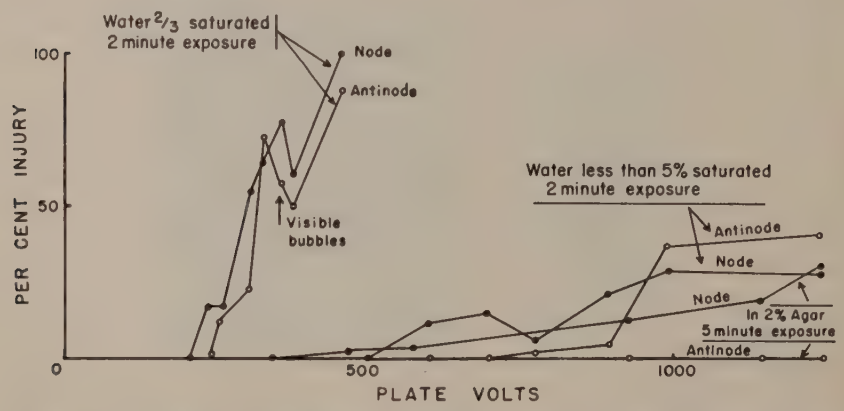

Fig. 5 Per cent of cells injured plotted against D.C. plate voltage of amplifier for Spirogyra filaments placed at pressure nodes and antinodes in various media (400 kc.).

with one-half at a pressure node and the other half at an antinode. The results (table 1) were in agreement with those given in figure 5. Under high pressure (150 psi) the damage distribution along vertically placed filaments was similar to that observed in degassed water or in agar gel (fig. 6), although the data are not as definite as those taken at atmospheric pressure due to difficulties in controlling the amount of dissolved gas at high pressure.

It is apparent that cavitation or its concomitants are highly damaging to living cells. Any procedure such as degassing, application of high pressure, or imbedding in a gel or a vis-

cous medium which tends to suppress cavitation also reduces greatly the degree of injury produced. However, the avoidance of cavitation then brings to light another source of injury associated with the acoustic particle motion of the medium.

TABLE 1

Injury to Spirogyra at node and antinode in water and in agar

PLATE VOLTS	INJURY IN WATER			INJURY IN AGAR			AGAR CONCENTRATION
	Exposure time	Per cent injured		Exposure time	Per cent injured		
		Node	Antinode		Node	Antinode	
	seconds			*minutes*			%
1000	10	74	80	5	49	0	1
1000	10	95	100	40	74	0	4
1000	25	56	75	50	40	0	2
1000	30	85	65	24	30	0	2
1400	5	70	78	0.41	17	0	2
1400	15	83	100	0.25	47	7	1

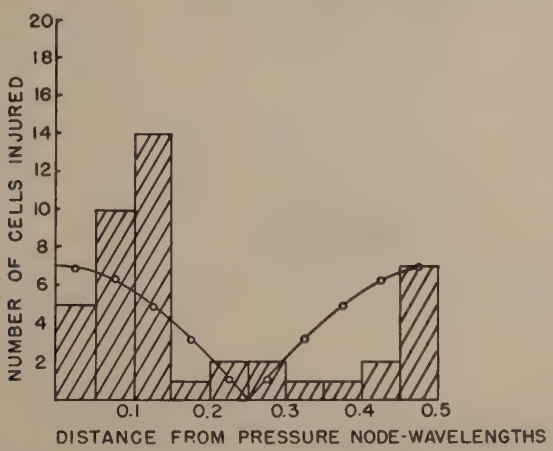

Fig. 6 Cell injury along sound field under hydrostatic pressure of 150 psi showing statistical distribution of injured cells. Smooth curve is a rectified sine wave scaled for same total number of injured cells (400 kc.).

If cavitation acts, as has been suggested, as a source of shock waves external to the cells, its effect should decrease as the cells are removed further and further from a cavitation region. Two halves of the same Spirogyra filament were

cast in a block of 2% agar whose upper boundary was the cover slip reflector. The pieces were held horizontally in a cage, on the lower surface of another cover slip placed in the agar at a pressure antinode, parallel to each other and about 5–7 mm apart. The agar beneath the cover slip was then cut away between the filaments leaving a channel several millimeters wide and so cut that the filaments were within the agar but a few tenths of a millimeter from the edge of the

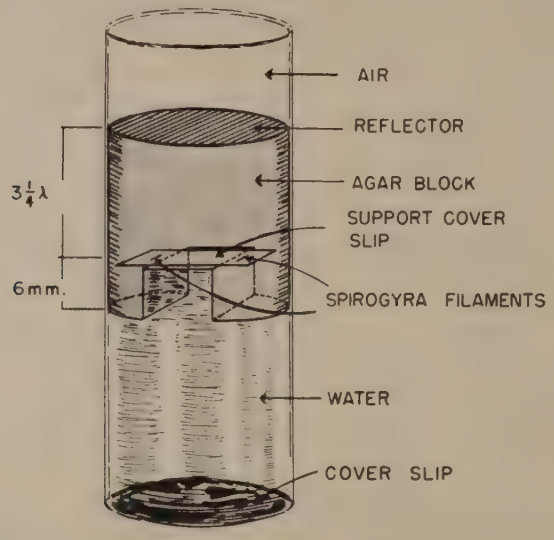

Fig. 7 Apparatus for determining relative injury as a function of distance from a cavitation region.

channel. The filaments were also at least 5 mm from the other edges of the block. The block was then placed in the water-filled cylinder and exposed to ultrasound (fig. 7). Cavitation occurred within the channel but not inside the agar. After exposure the number of injured cells in each piece was determined along with the lateral distances of the pieces from the channel edge. Similar experiments were carried out using erythrocytes and yeast cells suspended in the agar. The results (fig. 8) indicated that the injury produced decreases as the distance of the cells from the edge of the agar block

increased. It may be noted that the injury would be expected to fall off as the inverse square of the distance of a cell from a point source of lethal activity, as the inverse first power of the distance from a line source, and approximately logarithmically as the distance from a finite plane source whose plane includes the cells.

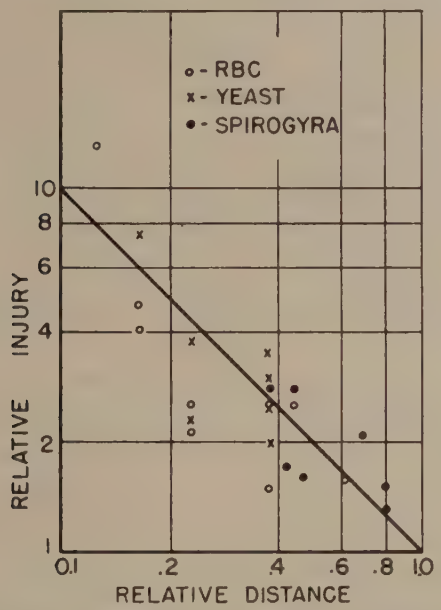

Fig. 8 Relative degree of injury of cells at a pressure antinode placed at various horizontal distances from a plane cavitation layer in water (400 kc.).

DISCUSSION

The results of the last experiment show that cavitation does act as an external source, presumably of shock waves, which produces injury by steadily bombarding the cells. It was suggested by Rayleigh ('17) that cavity collapse could give rise to shock pressures of many thousands of pounds per square inch. Since it is known that steady pressure of this order of magnitude can interfere with normal cell metabolic processes (Cattell, '36; Marsland and Brown, '36) it is conceivable that the action of cavitation is through the high

pressures produced. On the other hand, the injury produced at pressure nodes, in the absence of cavitation, must depend on some other mechanism, and since the type of injury is the same at both nodes and antinodes, it is desirable to look further.

There are several ways in which a sound field can produce effects in a liquid medium. There may be depolymerization of large molecules or oxidation. Molecular orientation may occur (Lucas, '39; Peterlin, '50) and colloidal particles may be subjected to turning moments (Burger and Söllner, '36). Small particles may be forced to move relative to the medium (Lamb, Hydrodynamics). Distortional oscillations of fluid particles may occur (Ackerman, '51). Heat production from sound absorption is also found especially in viscous media or at high particle or cell concentrations, although this factor is of little concern here since we are dealing with non-thermal effects.

It is helpful to be able to visualize the intra-cellular strains, or relative displacements of probable importance. Cells are not homogeneous. They contain a more or less visco-elastic cytoplasm with a variety of macro-molecules. They have a differentiated surface region and contain inclusions such as nuclei, vacuoles, etc., whose mechanical properties differ from those of their cytoplasmic medium. Mechanical forces destructive to these elements are potentially lethal.

It is not yet possible to assign a relative importance to any of these forces, nor to decide whether the damage to cells arises from a mechanical effect on the molecular elements, on the larger particles, or through a membrane change. It may be that different factors operate in different cells at different frequencies, or at different power levels. Evidently further experimental work is necessary.

SUMMARY AND CONCLUSIONS

1. Exposure of living cells to different parts of a standing wave sound field shows marked positional effects.

2. In the presence of cavitation, injury is widespread and rapid, and originates at pressure antinodes.

3. In the absence of cavitation, injury occurs slowly at the pressure nodes.

4. Mechanical injury to cells can occur in several ways but there is not yet enough information available to permit evaluation of the many factors involved.

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THE STIMULATION OF YEAST GROWTH AND RESPIRATION BY COMPOUNDS PRODUCED BY YEAST CELLS IRRADIATED WITH ULTRAVIOLET LIGHT [1]

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FIVE FIGURES

The ability of products from ultraviolet damaged cells to stimulate the growth and proliferation of normal yeast suspensions has been studied in some detail (Loofbourow, '48). Fardon, Carroll and Ruddy ('37) established that the oxygen consumption of living yeast cells was stimulated by direct ultraviolet and x-radiation. They showed that the addition of ultraviolet killed cells to living cultures produced respiratory stimulation. Fardon and Ruddy ('37) added centrifuged and filtered fluids from irradiated yeast to non-irradiated yeast suspensions. Respiratory stimulation by the cell-free fluid was pronounced, while that due to the washed cells was negligible.

Sizer and Loofbourow (see Loofbourow, '48), using centrifuged and filtered fluids from ultraviolet irradiated aqueous suspensions of *S. cerevisiae*, have increased both the endogenous and exogenous respiration of Baker's yeast. The effect has been blocked with cyanide. These preparations, tested for proliferation promoting effects on yeast cultures grown in

[1] Supported in part by a grant from the American Cancer Society, upon recommendation of the Committee on Growth of the National Research Council.

[2] Public Health Research Fellow of the National Cancer Institute.

[3] Deceased.

Reader's Medium ('27), showed marked stimulation of growth. In the presence of oxygen the growth promoting activity of injured cell products was 32 times that of un-injured cell products, while assays of the same preparations in non-aerated cultures showed unappreciable differences in activity.

Analyses of the fluids from irradiated yeast cells have been reported in detail. These fluids contain traces of amino acids and all of the known growth factors that are not photochemically labile. The latter contribute to the effects of these fluids on growth and O_2 consumption of suspensions of normal yeast studied in these experiments, but by no means account for all the observed effects (Loofbourow, '47; Webb and Loofbourow, '47).

The experiments reported here were undertaken in the hopes that kinetic studies of growth and respiration might offer some clue to the mechanisms and chemical agents involved in the effects produced on growth and metabolism by extracts of yeast cells damaged by ultraviolet light.

METHODS

(a) Methods of irradiation and preparation of fluids. Cell free suspension fluids from damaged and undamaged cells were prepared after the method of Loofbourow, Webb, Loofbourow and Abramowitz ('42). Washed, starch-free, moist yeast (*S. cerevisiae*, F. B. strain) was suspended at 100 gm (wet weight)/l of suspension in distilled water and divided into 2l samples. For the preparations from damaged cells, the experimental samples were stirred and irradiated with full ultraviolet from a low pressure quartz mercury arc for 8 hour periods. During these periods, the undamaged control samples were stirred at the same temperature. The damaged cells remained alive during most of the irradiation period, as determined by the methylene blue staining reaction, and some were capable of budding (Loofbourow, '42); they are not cytolyzed but have increased in permeability. Following irradiation, cell-free fluids were obtained by multiple centrifugation of the irradiated and non-irradiated cell sus-

pensions. The fluids were autoclaved at 15 pounds pressure for 20 minutes at pH 5–6. This method yields a sparkling-clear, straw colored supernatant, substantially free from protein.

(b) Measurement of growth and respiration. The effects on growth and respiration of the irradiated and non-irradiated cell products were studied on washed suspensions of *S. cerevisiae* (F.B. strain ATCC No. 7754) obtained from Sabouraud agar slants approximately 48 hours after inoculation. The cells were suspended in Reader's Medium containing 0.5% glucose. Respiration was measured in a Warburg constant-volume respirometer at 30°C. CO_2 was determined by the direct method of Warburg (Dixon, '43); the pH of the suspensions varied from 5.3 to 5.6 during the course of the experiments. The yeast concentration was measured with a calibrated photoelectric densitometer consisting of a Diafant Model O projector with an attached direct reading photocell.[4] Yeast was grown in square densitometer tubes selected for optical uniformity. The tubes were inserted in a large wheel at an angle of 17° producing a culture surface area of about 3 cm^2. The wheel rotated 64 cycles/min. in an incubated box at 30 $\pm$ 0.5°C. This technique effected a uniform mixing and aeration of the cultures. Growth rates were found to be equivalent in Warburg flasks and densitometer tubes.

RESULTS AND DISCUSSION

In figure 1 the growth, oxygen consumption and CO_2 production of yeast cultures grown in the presence of irradiated-cell supernatants (IS) and non-irradiated-cell supernatant (NS) are compared with cultures grown in an unsupplemented Reader's Medium (C). The data shown are typical of 7 independent experiments which yielded essentially similar results.

[4] Loofbourow and Dwyer ('38) have shown that this method has an inherent error of less than 10% as an estimate of wet weight, which varies with the age of the culture. After 8 hours of growth, we found the densitometer estimates of wet weight to be 5% greater than our direct measurements.

Table 1 contains the respiratory quotients (CO_2/O_2) calculated 90, 150, 210, and 270 minutes after the addition of glucose to the inoculated Reader's Medium. Data are presented for damaged cell products (IS), "undamaged" cell products (NS) and control.

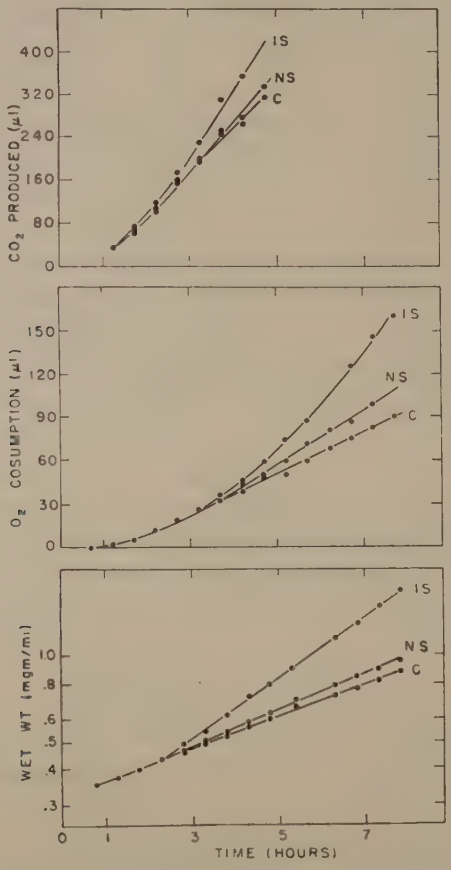

Fig. 1 Wet weight, O_2 consumption and CO_2 production vs. time.

C = cultures grown in unsupplemented Reader's Medium.

IS = Reader's Medium supplemented with supernatant fluid from irradiated cells.

NS = Reader's Medium supplemented with supernatant fluid from non-irradiated cells.

(0.02 ml IS or NS per milliliter suspension)

In figure 2 are shown the total O_2 consumption and dry weight increase of 8 hour cultures grown as in figure 1 and containing varying initial concentrations of radiation damaged cell products.

TABLE 1

Change of R.Q. with time for cultures grown in unsupplemented Reader's Medium (C) and in Reader's Medium supplemented with supernatant fluid from irradiated (IS) and non-irradiated cells (NS)

TIME	R.Q.		
Min.	IS	NS	C
90	8.3	7.5	7.5
150	8.4	7.3	7.0
210	7.0	6.7	6.2
270	5.3	5.1	5.9

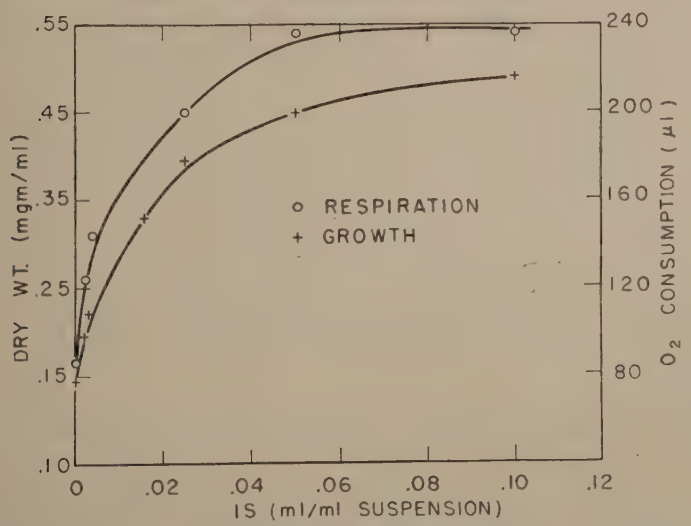

Fig. 2 Total 8 hour crops and oxygen consumption as a function of the initial concentration of supernatant from irradiated cells.

The effects of 0.001 M 2,4-dinitrophenol upon yeast suspensions grown in the presence and absence of radiation damaged cell products were studied with the results shown in table 2. Total growth and O_2 consumption were measured

380 minutes after the addition of glucose. Though growth is effectively blocked in all instances, the addition of supernatant fluid from irradiated cells continues to increase O_2 consumption of DNP-treated cells as compared with the DNP-treated controls.

That an intimate relationship exists between the increase of respiration and growth produced by the addition of products from radiation-damaged cells is implied by the data of figure 1, but this relationship is more cogently shown by

TABLE 2

Effect of 2,4-DNP [1] on total growth and respiration of 380 min. cultures

	GROWTH (MG/ML)		O_2 CONSUMP. μl O_2	
	No DNP	0.001M DNP	No DNP	0.001M DNP
I.S.	0.22	0.016	128.5	79.2
N.S.	0.104	0.0	121	62.2
C	0.073	0.0	111	49.0

[1] 2,4-dinitrophenol.

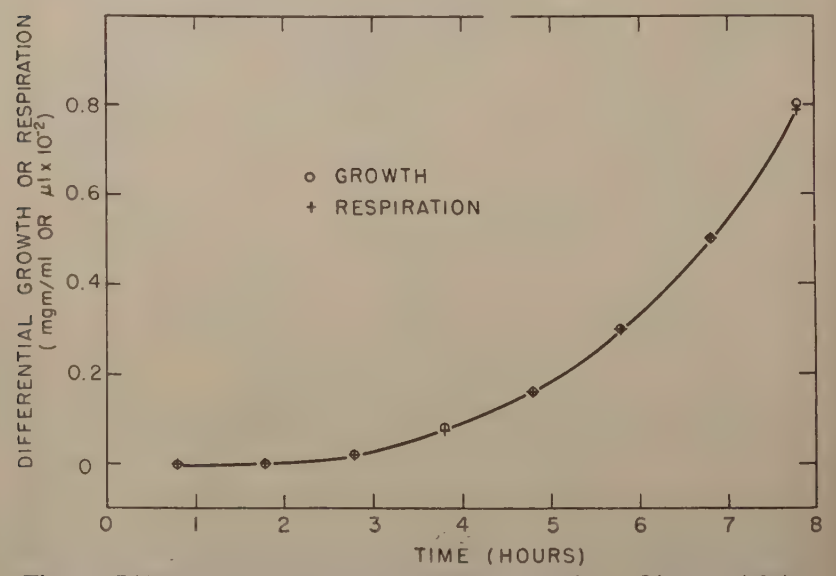

Fig. 3 Differential growth and respiration vs. time. [(wet weight)$_{IS}$ — (wet weight)$_C$ and (O_2 consumption)$_{IS}$ — (O_2 consumption)$_C$] vs. time.

figure 3 in which the differential growth and O_2 consumption (i.e., yeast concentration and O_2 consumption of "stimulated" suspensions minus the yeast concentration and O_2 consumption of simultaneous control suspensions), are plotted against time, with a proper adjustment of axes.

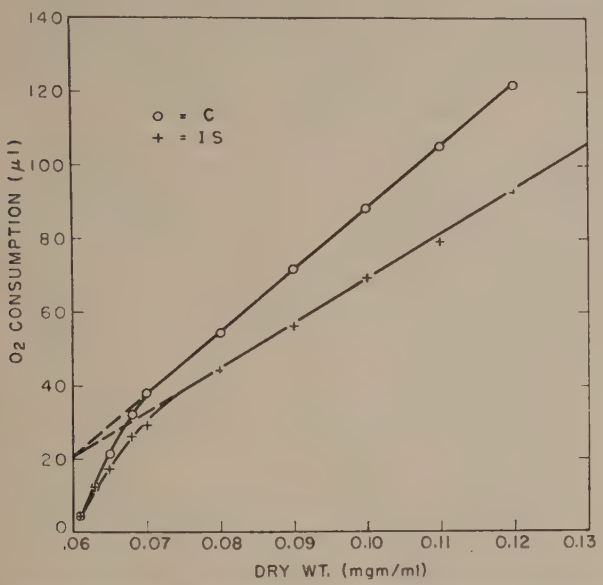

Fig. 4 O_2 consumption vs. dry weight of suspensions to which radiation damaged cell products have (IS) and have not (C) been added.

C = Cultures grown in unsupplemented Reader's Medium.

IS = Reader's Medium supplemented with supernatant fluid from irradiated cells.

This plot indicates that the differential growth and respiration due to the addition of radiation damaged cell products follow the same time course, which is apparently independent of the initial high respiratory activity associated with the early phase of the growth cycle. This is confirmed by figure 4 in which the O_2 consumption is plotted against the dry weight increase at various times throughout the growth cycle for suspensions to which products from radiation damaged cells have and have not been added. Extensions of the linear elements of the figure, which represent the situation

during the phases of active growth (log increase), cross the ordinate, or the point of zero weight *increase* (0.06 mg/ml), at the same value of O_2 consumption. This indicates that the initial "non-productive oxygen" consumption, in terms of weight increase, of both suspensions is probably equivalent and that differences in the *total* oxygen consumption during this period may be accounted for by the differences in the assimilation rate alone. Note that at the start the relative

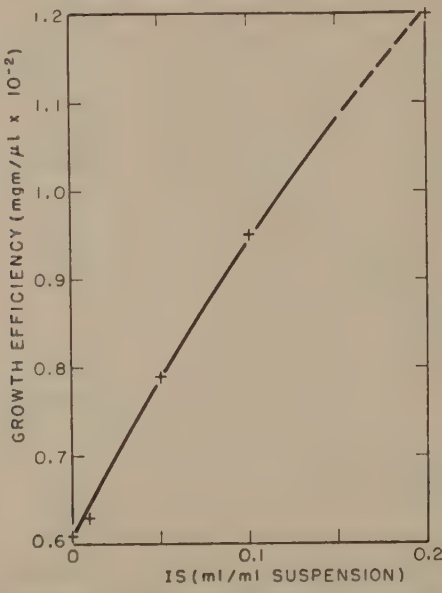

Fig. 5 Growth efficiency (wet weight increase per microliter O_2 consumed) vs. initial concentration of radiation damaged cell products (IS).

growth per unit oxygen consumed is the same, but soon becomes lesser in the control than in the experimental. However, from figure 4 it is evident that the efficiency of growth, in terms of dry weight increase per oxygen molecule consumed, is improved upon the addition of radiation damaged cell products. Changes of this efficiency with increasing concentrations of radiation damaged cell products are shown in figure 5. If one makes the simplifying assumption that a con-

stant fraction of the wet weight increase is due to assimilated carbohydrate as represented by the following equations:

$$a\ C_6H_{12}O_6 + bO_2 \rightarrow (CH_2O)n_1 + (b)\ CO_2 + bH_2O\ \text{(Control)}$$
$$c\ C_6H_{12}O_6 + dO_2 \rightarrow (CH_2O)n_2 + (d)\ CO_2 + dH_2O\ \text{(IS added)},$$

then for a constant weight increase $n_1 = n_2 = n$
by figure 4: $\frac{b}{n} > \frac{d}{n}$ or $b > d$,
it follows that $a > c$, where $6a = n + b$ and $6c = n + d$ or that $\frac{a}{n} > \frac{c}{n}$, which implies an increased efficiency in the presence of IS as concerns substrate utilization as well as O_2 consumption. In fact, larger crops per gram glucose utilized have been demonstrated by Mr. M. Abbate working in this laboratory ('50) lending support to this hypothesis. Moreover, analysis of the data of Mr. R. Kadel ('50) indicates that less nitrogen in the form of ammonium sulfate is utilized by growing cultures to which radiation damaged cell products have been added than to those to which they have not been. A plot of the growth efficiency (weight increment O_2 consumed) vs. the IS concentration from our data (fig. 5) shows a striking resemblance to the study of Burke and Lineweaver ('30) in which efficiency is plotted as a function of limiting NH_3 nitrogen concentration in Azotobacter cultures. Increased crops per unit O_2 consumed or per unit glucose oxidized might therefore be due to the amino acid content of the fluids from irradiated cells as well as to known and unknown growth factors which may be present.

SUMMARY

1. Products from cells damaged by ultraviolet radiation increase the oxygen consumption and carbon dioxide production of Baker's yeast more than do products from undamaged cells and the oxygen consumption of both its greater than that of the control cultures.

2. In the presence of 0.001 M 2,4-dinitrophenol, the oxygen consumption of suspensions to which damaged cell products have been added is greater than that of controls, though growth of both cultures is effectively blocked.

3. The oxygen consumption is intimately associated with corresponding growth increases and follows the same time course.

4. The assimilation is accomplished by considerable oxygen sparing, in terms of oxygen consumption per wet weight increase, in the presence of damaged cell products as compared with controls, indicating that these products promote the more efficient utilization of oxygen by growing yeast cultures.

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THE USE OF DIALYZED MEDIA FOR STUDIES IN CELL NUTRITION

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FOUR FIGURES

INTRODUCTION

The potential value of tissue culture in the field of cell nutrition has been apparent since the early days of its development by Harrison, Carrel and other in the years following 1912. Progress has been slow, however, owing to the difficulty in obtaining a defined basal medium for growth studies. The components which have been used for the cultivation of animal cells — plasma, serum, and tissue extracts — are complex, unknown, and variable in composition. It has proved exceptionally difficult to find satisfactory substitutes for these in terms of known pure substances, although significant advances have been made in the last several years. Earle and co-workers have developed methods for cultivating cell suspensions beneath perforated cellophane or on bare glass (Evans and Earle, '47; Evans, Earle, Sanford, Shannon and Waltz, '51; Sanford, Earle, Evans, Waltz and Shannon, '51; Earle, Sanford, Evans, Waltz and Shannon, '51; Shannon and Earle, '51) thus dispensing with a plasma coagulum. Serum and embryo extract are retained in the supernatant fluid. Other investigators, notably White ('46, '49a) and Parker and associates (Morgan, Morton and Parker, '50, '51; Morton, Morgan and Parker, '50, '51; Parker, Morgan and Morton, '50; Parker, '50; Morgan, Morton, Healey and Parker,

[1] Aided by grants from the Cancer Research Coordinating Committee, University of California.

'51) have devised completely synthetic media from known crystalloidal growth factors, culturing tissue fragments on bare glass in roller tubes. The nutrient fluids contain salts, amino acids, vitamins and other known pure substances. Indefinite cultivation under these conditions has not been possible although by progressive refinements and additions the survival time has been increased from a few days to two months or more.

A critical evaluation of synthetic media at their present level of evolution is provided by the recent work of Gerarde, Jones, Wang, Wilson, and Winnick ('51), Gerarde, Jones, and Winnick ('52), and by the unpublished experiments of Evans, Shannon, Waltz, Bryant, Sanford and Earle ('52). Gerarde and co-workers cultured chick tissues in roller tubes. At varying times of incubation, tubes were removed and their protein content determined by direct isolation and weighing. Extensive protein synthesis occurred with tissues grown in 25% embryo extract, but not with the synthetic media tested. Evans et al. employed cell suspensions of mouse fibroblasts in flasks; nuclear counts were made as an index of growth. Over a period of 11 days the number of cells increased prominently in cultures containing horse serum and embryo extract, while cultures in White's medium as well and the Morgan and Parker nutrient (no. 199) showed a conspicuous decline in cell count from the starting value. These results suggest that the synthetic nutrients currently available do not promote growth and permit only a limited survival of cells *in vitro*.

Recognizing the difficulties inherent in devising a completely synthetic medium, Fischer ('41) approached the nutritional problem in a novel way. Instead of synthetic mixtures he utilized plasma, embryo extract and serum, but simplified each by separate dialysis prior to final combination. Ringer's solution fortified with 0.1% glucose served as a dialyzing fluid and was not changed during the 8-day dialysis period. The object of dialysis was to remove or reduce the concentration of diffusible nutrient factors (e.g.— amino acids)

while retaining proteins and other large molecules to form a basal medium. Fischer and co-workers have repeatedly found this basal medium inadequate to support outgrowth of chick fibroblasts, the margins of the explants undergoing disintegration within 24 hours (Fischer, '41, '46, '48; Fischer, Astrup, Ehrensvard and Oehlenschlager, '48; Ehrensvard, Fischer and Stjernholm, '49). According to Fischer, this disintegration results from removal through dialysis of amino acids and other factors essential for survival and growth of the cells in question. This assumption forms the cornerstone of an extensive series of investigations in which disintegration in Ringer-dialyzed media has been prevented and outgrowth restored by supplements of known pure growth factors (summarized by Fischer, '46; Fischer et al., '48; Ehrensvard et al., '49; and in review articles by others: Nutrition Reviews, '47, '49, '50, Swanson and Clark, '50; Holter, '50).

The experiments of Fischer and his collaborators suggest that dialyzed media may provide valuable assistance in characterizing the specific nutritional factors as yet unknown which are required for progressive growth and maintenance of cells *in vitro*. The specific assumptions, however, which underlie Fischer's work and his interpretations therefrom lack confirmation from other laboratories, and have been brought into question by White ('49b), the only investigator up to the present time who has published a repetition of Fischer's basic experiments. White found that simple dilution of Ringer-dialyzed media with Tyrode solution prevented disintegration and partially restored outgrowth of chick bone fibroblasts, without further supplementation. This result is in harmony with the experiments to be described in the present paper which are concerned with a general evaluation of dialyzed media as a tool for nutritional studies.

MATERIAL AND METHODS

Media. All cultures were made with chicken plasma, chick embryo extract, and horse serum. Embryo extract (EE_{50})

was prepared from 12-day embryos by adding equal amounts by weight of Gey's solution and grinding thoroughly in motor-driven all glass homogenizers. The resulting homogenates were centrifuged at 3000 RPM for 30 minutes and the supernatants stored at 4°C. in rubber stoppered tubes. Sterility tests on homogenates were carried out by incubating the centrifuged sediments for 24 hours at 38°C. followed by inoculation into N.I.H. thioglycollate broth (Difco) for 7 days. Chicken plasma was obtained by heart puncture from cockerels 6–9 months old. Enough heparin (Connaught Laboratories) was included to give a final blood concentration of approximately 0.003%. Horse serum was obtained commercially from Cutter Laboratories, Berkeley, and sterilized in large batches by passage through a Selas filter, porosity no. 015.

Dialysis procedure. Plasma, serum, and embryo extract were dialyzed in large (21.) Erlenmeyer flasks by a modification of the method first devised by Fischer ('41). Each unit included a short stem of glass tubing which passed through a rubber stopper in the mouth of the flask, and to which was affixed by string an appropriate length of dialysis tubing (Visking Corp., New York; diameter ¾ inch when round). The lower end of the tubing was closed by a double knot. The empty dialysis sac was lowered into an Erlenmeyer flask filled with distilled water and the entire unit sterilized by autoclaving at 15 lbs. for 20 minutes.

For dialysis 25 ml portions of plasma, serum, and embryo extract were introduced by pipette into separate units and the glass tubes closed with rubber stoppers. Each unit was then transferred by lifting into a flask of dialyzing solution and stored at 8°C. Except in a few preliminary experiments to be noted, the dialyzing fluid was changed daily (except Sunday) for 8 days. Aseptic precautions were observed throughout. The media were then removed by pipette, centrifuged, and stored at 4°C. in rubber stoppered tubes. Routine sterility tests were made by inoculating 0.1 ml from each

dialysis unit in N.I.H. thioglycollate broth (Difco). Only freshly dialyzed media were used for experiments.

Culture methods. All cultures were made in Carrel D-3.5 flasks using fresh explants from 12- or 16-day chick heart. The explants were obtained from the ventricular region and cut to uniform size (approximately 1.5 mm diameter) with Bard-Parker No. 10 scalpels. At the time of culturing each flask received 0.5 ml chicken plasma to which 1.0 ml clotting fluid was added and a single explant appropriately positioned just prior to coagulation. Subsequently 1.0 ml supernatant was added and the cultures sealed with rubber stoppers. For the majority of cultures the supernatant fluid contained 40% horse serum, 15% chick embryo extract (EE_{50}) and 45% glucose-free Gey's solution. In a few of the earlier series 20% embryo extract was used. In the saline fraction the following were incorporated isotonically so as to give the corresponding concentrations in the completed medium: 0.15% D-glucose, 0.002% phenol red, 100 units/ml sodium penicillin G (Merck) and 50 units/ml dihydrostreptomycin (Pfizer). Serum was omitted from the clotting fluid and the concentration of other constituents increased 50% to match levels in the clot with those of the supernatant.

All cultures were incubated at 38°C. At biweekly intervals the fluid phase was removed and fresh supernatants added without washing the cultures. Routine data on areal spread were obtained by tracing the cultures at 15 × with a projectoscope. Areal increases (final area minus initial area) were determined with a planimeter and the results treated statistically.

EXPERIMENTAL WORK

Outgrowth in Ringer-dialyzed media

Preliminary studies. Exploratory experiments were carried out with cultures made in hanging drops, roller tubes, and Carrel flasks. In confirmation of Fischer's observations ('41) excellent outgrowths developed from chick heart explants in normal undialyzed media but were essentially absent in media prepared by dialysis against Ringer-glucose solution.

If the dialyzing fluid was not changed, as in Fischer's original experiments, outgrowth in the resulting media was for the most part absent, although in a few cultures a narrow migratory zone developed (see plate 1, fig. 2). If the dialyzing fluid was changed at least three times during the 8-day dialysis period, there was never any outgrowth in the resulting medium. Only disintegrating cells were found on the edges of the explants at 24 hours.

Additional investigation showed that outgrowth in Ringer-dialyzed media could be restored by the addition of dialysate from chick embryo extract. This result shows clearly that

TABLE 1

Sample pH changes in media dialyzed 8 days against Ringer-glucose solution

	METHOD OF DIALYSIS	CHICKEN PLASMA	CHICK EMBRYO EXTRACT	HORSE SERUM
Initial pH	....	8.0	7.0	7.9
pH after dialysis	Dialyzing fluid not changed	6.6	5.9	7.0
	Dialyzing fluid changed daily	5.9	5.4	6.5

All measurements made with Beckman pH meter, Model G.

dialysis in itself does not cause toxic alterations of proteins or other components of the medium.

pH changes during dialysis. Further analysis revealed that the inadequacy of Ringer-dialyzed media for outgrowth could be correlated with a pronounced fall in pH level during the dialysis procedure. These facts were established by direct measurements with a Beckman pH meter and are illustrated by table 1. A progressive decline is evident in all cases, with a greater drop if the dialyzing fluid is changed daily. Since Ringer-glucose solution is unbuffered, this fall in pH appears to reflect the progressive removal of bicarbonate, phosphate, and other diffusible buffers initially present. If the isoelectric points of the residual proteins were predominantly on

the acid side of neutrality, pH levels similar to those observed would be expected.

Treatment of media with CO_2 mixtures. In the experiments of Fischer and co-workers all cultures in dialyzed media were exposed to a mixture consisting of 8% CO_2, 80% O_2, and 12% N_2 with the objective of adjusting the final pH of the medium to 7.0–7.2. Gassing with CO_2 is a routine procedure in many laboratories and on undialyzed media gives excellent results (see Parker, '50). Cultures made with such media are ordinarily alkaline when first prepared and treatment with CO_2 mixtures lowers the pH effectively to physio-

TABLE 2

Effect of gassing on pH of media in plasma-coated roller tubes

PLASMA AND SUPERNATANT [1]	INITIAL pH OF SUPERNATANT	pH AFTER GASSING 2 MIN. WITH		
		1% CO_2	5% CO_2	8% CO_2
Not dialyzed	8.1	7.9	7.2	7.0
Dialyzed [2]	7.8	6.6	6.2	6.0

[1] Composition of superantant: 40% horse serum, 40% Gey's saline, 20% chick embryo extract (EE_{50}).

[2] Dialyzed 8 days against Ringer-glucose solution; dialyzing fluid changed daily.

[3] All gas mixtures contained 80% oxygen in addition to CO_2; residue N_2.

logical levels. The picture is different, however, for Ringer-dialyzed media which already exhibit a low pH (table 1). In this case gassing with CO_2 does not adjust the reaction of the medium to physiological values. Experiments supporting this point are summarized in table 2. Roller tubes were coated internally with dialyzed or undialyzed plasma and after clotting 1.0 ml of the corresponding supernatant fluid was added. The tubes were then gassed for two minutes with an appropriate mixture of CO_2, O_2, and N_2, re-sealed with rubber stoppers, and allowed to equilibrate for a few minutes at room temperature. For pH measurements supernatants from groups of three tubes were pooled and values obtained with a Beckman pH meter. All figures listed in table 2 represent the average of two separate determinations.

These data confirm the effectiveness of CO_2 mixtures in lowering the pH of undialyzed media to desirable levels. It is also apparent that treatment with CO_2 does not raise the pH of Ringer-dialyzed media but rather causes a further decline to values well below the physiological zone.

Elevation of pH with Na_2CO_3. Although CO_2 mixtures do not raise the pH of Ringer-dialyzed media it is possible to elevate the reaction directly by the addition of $NaHCO_3$ or Na_2CO_3. Experiments of this type were performed with chicken plasma, chick embryo extract, and horse serum dialyzed exactly as described by Fischer ('41) without change of dialyzing fluid. Prior to culturing, however, a convenient volume of each was adjusted to pH 7.4–7.6 by the addition to 1.7% Na_2CO_3 in the presence of 0.002% phenol red. Final pH levels were verified with a Beckman pH meter.

Cultures in Carrel flasks were prepared with the carbonate-adjusted media, and were compared with others made up from non-adjusted media. Some of the latter were gassed with 8% CO_2. An additional group of cultures was set up using carbonate-adjusted plasma clotted with embryo extract, but with saline only (Gey's solution) as a supernatant fluid. The typical results of this experiment are illustrated by text figure 1 and plate 1, figures 2–4. Without the addition of Na_2CO_3 no outgrowth developed or at most a negligible fringe similar to that shown in the picture. Cultures gassed with 8% CO_2 showed no outgrowth whatever. By contrast explants in media adjusted to pH 7.4–7.6 with Na_2CO_3 exhibited and even, sustained outgrowth. During the first days of culture the cells were comparatively free of undue fat or granular inclusions although later a marked vacuolization occurred. Substitution of saline for the usual serum-embryo extract supernatant resulted in a decreased level of outgrowth. Similar results were obtained when the initial pH adjustment was made with 1.4% $NaHCO_3$ instead of Na_2CO_3. In either case the explants could be subcultured at the end of the 8-day initial period and exhibited renewed outgrowth in the fresh medium.

Role of bicarbonate. The action of sodium carbonate or bicarbonate in restoring outgrowth includes a direct effect in addition to the simple elevation of pH from demonstrably low initial levels. This finding emerged from experiments in which 0.15 N — NaOH was used to elevate the final pH of Ringer-dialyzed media to 7.4–7.6. Outgrowth did not occur in these media although if additional bicarbonate was added a flourishing outgrowth resulted. In a subsequent series of

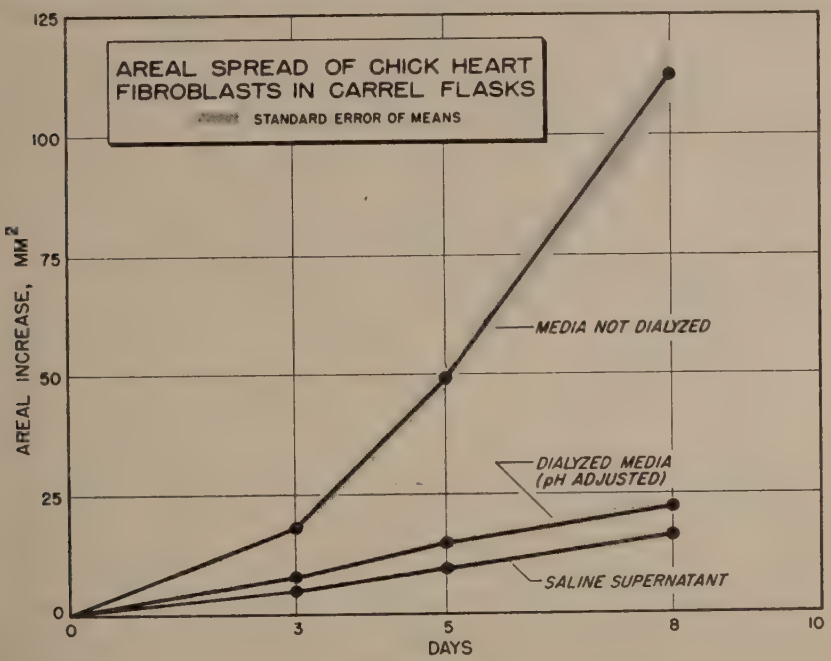

Figure 1

experiments cultures were set up with graded levels of added bicarbonate. The results are shown in table 3. These indicate clearly that outgrowth in Ringer-dialyzed medium is dependent on a minimal level of added bicarbonate with areal increases paralleling bicarbonate concentration above this point.

It seems probable that low bicarbonate levels may account at least in part for the failure of outgrowth in Fischer's experiments. His published data (Fischer et al., '48; Ehrens-

vard, Fischer and Stjernholm, '49) suggest that supplemental bicarbonate represented 0.05% or less in the final culture medium. The change in CO_2/bicarbonate ratio with pH would make this deficiency increasingly critical at levels of pH 7.0 or less as used in his experiments.

Role of phosphate. The dialyzing fluid, Ringer-glucose, lacks phosphate as well as bicarbonate and the question arises whether supplementary phosphate influences outgrowth as does bicarbonate. The data of table 3 provides a partial answer. In these experiments the saline used was buffer-free, and consequently the media contained no supplementary phosphate. Addition of 0.001 M-phosphate to the culture medium

TABLE 3

Effect of bicarbonate on outgrowth of chick heart fibroblasts in Ringer-dialyzed media

ADDED $NaHCO_3$, GM/100 ML [1]	AREAL INCREASE [2] PER EXPLANT AT 9 DAYS, MM^2
0.005	No outgrowth
0.05	7.6 ± 1.4
0.10	29.5 ± 2.4
0.20	51.1 ± 7.0

[1] Supplementary phosphate omitted from culture medium.

[2] Mean value and standard error (Snedecor, '46). Six cultures in each series.

did not alter the appearance of the cells or significantly change the areal spread. Concentrations of 0.003 M-phosphate or more were definitely inhibiting. It is apparent that for short-term experiments at least, supplementary phosphate is not a limiting factor in Ringer-dialyzed media except at toxic levels. Inorganic phosphate is presumably made available from other forms by phosphatase activity, as Hanks ('49) has previously shown for undialyzed media.

Modification of dialysis procedures

Comparison of dialyzing fluids. The results outlined in the preceding sections indicate the need for altering the original dialysis procedures of Fischer ('41) to provide adequate

control of pH and buffer strength. Accordingly we have compared the properties of media dialyzed against several different salines. The composition of these fluids is shown in table 4. The list includes besides Gey's and Ringer's solutions a saline termed F-2 which has been devised for the present experiments by modification of Gey's fluid. The bicarbonate and phosphate concentrations have been increased in F-2 solution and the pH level as freshly prepared falls between 7.7 and 7.8. All of the solutions used were sterilized

TABLE 4

Composition of dialyzing fluids[1]

	F-2 SOLUTION	F-2 SOLUTION, PHOSPHATE-FREE	GEY'S SOLUTION, SUGAR-FREE	RINGER'S SOLUTION
NaCl	7.33	7.50	8.17	9.00
KCl	0.38	0.38	0.38	0.42
$CaCl_2$ Anhydrous	0.13	0.13	0.13	0.25
$MgCl_2 \cdot 6H_2O$	0.21	0.21	0.21	...
$NaHCO_3$	1.40	1.40	0.25	...
KH_2PO_4	0.11	...	0.025	...
$Na_2HPO_4 \cdot 12H_2O$	...	...	0.30	...
Na_2HPO_4 Anhydrous	0.30	...	...	...
Distilled water	1000 ml	1000 ml	1000 ml	1000 ml

[1] Amounts of salts are given in grams.

by vacuum filtration through a 2 × 10 inch Selas microporcelain candle, porosity no. 015, and the sterile saline stored in 21.–Erlenmeyer flasks with rubber stoppers.

Media dialyzed against Ringer's or Ringer-glucose solution are much paler than undialyzed media even when later corrected for pH drop and small changes in volume during dialysis. Besides this characteristic loss of color the chick embryo extract exhibits a greater opacity after dialysis. A variable precipitation occurs during dialysis with Ringer's solution and is especially marked with embryo extract. The precipitate does not redissolve in buffered saline. These observations suggest that the pH drop accompanying dialysis

against Ringer's solution leads to denaturation in part of media proteins. By contrast media which are dialyzed against F-2 solution take on the pH and buffer levels of the dialyzing fluid, retain their original color and appearance, and exhibit less precipitation than undialyzed media stored for a similar interval in the refrigerator at 4°C. Media dialyzed against F-2 without phosphate, or against sugar-free Gey's solution were similar in appearance to those prepared with F-2. It

TABLE 5

Influence of dialyzing fluid on outgrowth of chick heart fibroblasts in dialyzed media [1]

DIALYZING FLUID [2]	AREAL INCREASE [3] PER EXPLANT AT 10 DAYS	% DIFFERENCE FROM F-2	SIGNIFICANCE OF DIFFERENCE
	mm^2		
F-2 solution	90.1 ± 8.9	..	..
F-2 solution without phosphate	88.2 ± 5.5	— 2.1	$P = > 0.8$
Gey's solution, sugar free	95.7 ± 10.8	+ 6.2	$P = > 0.6$
Ringer's solution	56.2 ± 4.1	— 37.6	$P = < 0.01$

[1] All cultures adjusted to contain final concentrations of 0.21% $NaHCO_3$, 1.25×10^{-3} M — supplemental phosphate, 0.002% phenol red, 0.15% D-glucose, 100 units/ml sodium penicillin (Merck), and 50 units/ml dihydrostreptomycin (Pfizer).

[2] See table 1 for composition of dialyzing fluids.

[3] Mean value and standard error (Snedecor, '46). Six cultures in each series.

is thus apparent that lowering of pH rather than reduction of bicarbonate or phosphate is responsible for the changes noted with Ringer dialysis.

Assays in vitro. Cultures were prepared using media dialyzed against each of the fluids described in the last section. Prior to culturing the concentrations of bicarbonate, phosphate and glucose in the various media were adjusted to a common level. Comparative data were obtained on areal increase, appearance of cells and colonial growth patterns. Table 5 presents a summary of the areal changes. If the level of outgrowth in media dialyzed against F-2 is taken as a yardstick, it appears that reduction of both bicarbonate

and phosphate in the dialyzing fluid (Gey's solution) or even the elimination of phosphate (phosphate-free F-2) does not significantly influence areal spread, provided that phosphate and bicarbonate are adjusted to a similar level before culturing. On the other hand, the level of outgrowth in media dialyzed against Ringer's solution fluid is conspicuously less than with any of the others. This differential is regularly reproducible and is also found if Ringer-glucose is used in place of Ringer alone.

Microscopic studies showed that regardless of the dialyzing fluid used, characteristic differences separate cultures in dialyzed from those in undialyzed media. In addition to a reduced surface area, these include a looser, less regular ("brush-heap") arrangement, rather than radial orientation of cells in the growth zone. Vacuolation occurs earlier and is more intense, with a tendency for the original explant to remain recognizable for long periods instead of rapidly breaking down to become an indistinguishable part of the outgrowing ring of cells.

In media prepared with F-2 solution the outgrowth was clear and relatively free of inclusions during the initial days following culture. Subsequently the cells tended to enlarge, to become filled with small to medium vacuoles, and to develop a brown, refractile appearance. The outgrowth zone continued to expand as these changes occurred and if fragments of the marginal zone were subcultured progressive outgrowth took place for three successive generations tested. Outgrowths with Gey-dialyzed media were identical with those just described for F-2. On using phosphate-free F-2, the vacuolation and refractile condition of the corresponding cultures were further accentuated. Outgrowths in Ringer-dialyzed media were less extensive, with a more open, net-like arrangement of the cells than with F-2 media. The cells had fewer but larger vacuoles than with F-2 media and commonly showed more jagged outline, especially at the periphery. The pH drop in culture was regularly less with cultures in Ringer-dialyzed media than with cultures in F-2 media. Al-

though quantitative data are not available, these facts suggest that the number of cells may be less in cultures made with Ringer media than with F-2 media.

Outgrowth in F-2 media of varied composition

To use dialyzed media effectively for nutritional studies it is essential to establish the levels of outgrowth which occur reproducibly in cultures unsupplemented with nutrients except for sugar and inorganic salts. Since Fischer and associates failed to obtain outgrowth under these conditions, their extensive experiments do not afford the desired information. Baseline experiments were accordingly carried out using F-2 dialyzed media and the results are summarized in table 6. To avoid confusion in comparing these figures with those of table 5 it should be emphasized that the level of bicarbonate used in culture differs in the two cases. The influence of bicarbonate on areal spread has been described in a preceding section of the present paper. In the figures presented in table 6, increase in area is related in each case to the level of outgrowth in a basal medium containing 40% dialyzed horse serum, 15% dialyzed embryo extract (EE_{50}), and 45% Gey's saline. Omission of horse serum from the supernatant resulted in relatively large increases in area during the first few days of culture but the rate of increase fell off after a week so that the 10-day areas were not significantly different from the basal level. The cells in these cultures were conspicuously more refractile and vacuolated than those in the basal medium. This difference may reflect the presence of essential proteins in horse serum similar to the non-dialyzable factors recently described by Jacquez and Barry ('51) in human placental cord serum, and found by them necessary for active growth of fibroblasts.

Paradoxically, the substitution of chicken serum for horse serum gave an inferior outgrowth. Cultures made with dialyzed chicken serum showed a somewhat smaller areal increase and a lesser pH drop over a 19-day period, and the

TABLE 6

Outgrowth of chick heart fibroblasts in media prepared by standard dialysis [1]

TYPE OF MEDIUM	COMPOSITION OF SUPERNATANT PER CENT [2]			AREAL INCREASE [3] PER EXPLANT AT 10 DAYS	PER CENT DIFFERENCE FROM OUTGROWTH IN BASAL MEDIUM	SIGNIFICANCE OF DIFFERENCE
	EE_{50}	Serum	Gey's solution			
				mm^2		
Basal medium	15	40	45	54.2 ± 5.9	...	....
Serum omitted	15	..	85	52.5 ± 2.7	— 3.1	$P = > 0.9$
Chicken serum instead of horse serum	15	40	45	38.8 ± 5.0	— 28.4	$P = 0.4$
EE_{50} reduced	0.4	..	59.6	16.5 ± 3.1	— 69.6	$P = < 0.001$
EE_{50} reduced; serum omitted	0.4	..	99.6	15.4 ± 1.3	— 71.6	$P = < 0.001$
EE_{50} intensively dialyzed	15	40	45	46.1 ± 2.7	— 14.9	$P = 0.25$
Undialyzed media	15	40	45	128.7 ± 1.9	+ 137	$P = < 0.001$
Undialyzed media; EE_{50} reduced	0.3	40	59.7	42.3 ± 3.4	— 22.0	$P = 0.1$

[1] Dialysis against F-2 at pH 7.7 according to procedure described in text.

[2] All cultures with dialyzed media adjusted to contain 0.15% D-glucose, 0.085% $NaHCO_3$, 0.002% phenol red, 100 units/ml sodium penicillin G (Merck), and 50 units/ml dihydrostreptomycin (Pfizer).

[3] Mean value and standard error (Snedecor, '46). Six cultures in each series.

cells became intensely vacuolated. The basis for this effect is not yet clear.

The data of table 6 also show that the concentration of dialyzed embryo extract has a marked influence on area spread. Reduction of EE_{50} to 0.4% greatly decreases the surface area. Somewhat lower values were obtained by omitting EE_{50} completely and using thrombin as a clotting agent (Parke-Davis, 20 units/ml in final clot). At this level, however, the thrombin preparation was inhibiting even in the presence of EE_{50} so that a direct comparison of areas was not possible. The activity of embryo extract does not disappear with more intensive methods of dialysis. A 5 ml sample of EE_{50} was dialyzed 8 days on a Boerner shaker against many two-liter changes of F-2. After correction for a 4-fold volume change the resulting extract still displayed a prominent level of activity (see table 6). These data support the concept of both dialyzable and non-dialyzable growth factors in chick embryo extract as maintained by Fischer ('46) and others. Finally the data at the end of table 6 on cultures in undialyzed media form an additional frame of reference. It is apparent that dialysis reduces the level of outgrowth both in cultures containing minimal amounts of embryo extract as well as in those containing relatively high levels of EE_{50}.

DISCUSSION

The results presented in this paper confirm the usefulness of dialyzed media for nutritional studies as first conceived by Fischer ('41). When media dialyzed against a buffered saline are used for culture the resulting outgrowths exhibit a characteristic syndrome of deficiency in areal spread, cell appearance, and colonial pattern when compared to control cultures in undialyzed media. These conspicuous deficiencies can be obliterated by the addition of dialysate from chick embryo extract and a sensitive assay is thus afforded for the active growth factors contained therein. The results of these experiments suggest that one of the most promising

avenues of approach to an eventual synthetic medium lies in the fractionation of chick embryo dialysate, using dialyzed media as a basis for assay. On the other hand a consideration of the data presented here suggests that a re-orientation of earlier experiments is clearly required if dialyzed media are to provide useful information in cell nutrition. This point requires some emphasis because the nutritional studies of Fischer and co-workers (summarized by Fischer, '46; Fischer et al., '48; Ehrensvard, Fischer and Stjernholm, '49) are based on the failure of outgrowth in Ringer-dialyzed media. They have consistently maintained that failure of outgrowth stems from a primary deficiency of essential amino acids and other nutrients, and that the specific nature of these may be ascertained by determining which substances are capable of preventing disintegration and restoring outgrowth when added as supplements to the dialyzed medium. The present experiments demonstrate, however, that a stable and continuing outgrowth can be produced in Ringer-dialyzed media without the addition of further nutrients of any kind. A reasonable doubt is thus cast on the validity of conclusions relative to the role of specific amino acids or other nutritional factors studied in Fischer's experiments. It seems clear that in order to determine the true significance of these earlier investigations, a re-evaluation in terms of pH and buffer capacity will be necessary.

The present experiments also separate more clearly than heretofore the relative importance of the various non-dialyzable components for outgrowth in the standard plasma-serum-embryo extract medium. The data presented show that an initial outgrowth of chick heart fibroblasts can take place in a medium consisting of dialyzed plasma alone plus a saline supernatant, without added serum or embryo extract. The narrow rim of cells appearing under these conditions represents a minimal response. If dialyzed horse serum is added to the supernatant the areal increase over a 10-day period is identical and because of the sparse character of the outgrowth it is evident from direct observation that the number of cells

in the marginal zone is substantially the same as with plasma alone. The addition of dialyzed embryo extract leads to the development of a much larger outgrowth zone with conspicuous and obvious increases in number of cells in this region over a 10-day interval, with or without the presence of serum. Dialyzed embryo extract does not lose this activity even when intensively dialyzed for long periods on a shaker. It should be emphasized, however, that the comparisons made here deal solely with effects on the composition and extent of the outgrowth zone bordering the original explant and may not necessarily parallel other growth indices, e.g.— changes in protein content or nuclear count.

ACKNOWLEDGMENT

It is a pleasure to acknowledge the capable assistance of Mr. Albert Benedict, Mr. Ronald Branson, Mr. John Fordon, Mr. Robert Janson, Miss Miriam Lieberman, and Mr. Charles Oakes in executing the experiments described in this paper.

SUMMARY

1. Chick heart fibroblasts have been cultivated in chicken plasma, horse serum and chick embryo extract previously subjected to dialysis against balanced salt solutions. Variations in dialysis procedure and composition of media have been analyzed to evaluate the role of dialyzed media as a tool in nutritional research.

2. Evidence is presented which indicates that the failure of outgrowth in Ringer-dialyzed media stems from a fall in pH and reduction in bicarbonate during dialysis, rather than from a nutritive deficiency *per se* as maintained by previous investigators. A stable and continuing outgrowth of chick heart fibroblasts occurs in Ringer-dialyzed media following adjustment of pH and bicarbonate to physiological levels.

3. Methods are outlined for routine aseptic dialysis of media at constant pH and buffer strength. Assays with chick heart fibroblasts indicate that dialysis of media under these conditions reduces but does not abolish outgrowth.

4. Outgrowths of chick heart fibroblasts in media dialyzed against buffered salines are clearly suboptimal when compared with similar outgrowths in undialyzed media. Differences noted include reduction in surface area as well as characteristic alterations in the appearance of cells and colonial pattern.

5. The deficiency syndrome produced by dialysis can be eliminated by adding the dialyzable fraction of chick embryo extract to the dialyzed medium. A sensitive assay is thus provided for analysis of active factors in the dialysate fraction.

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PLATE

PLATE 1

EXPLANATION OF FIGURES

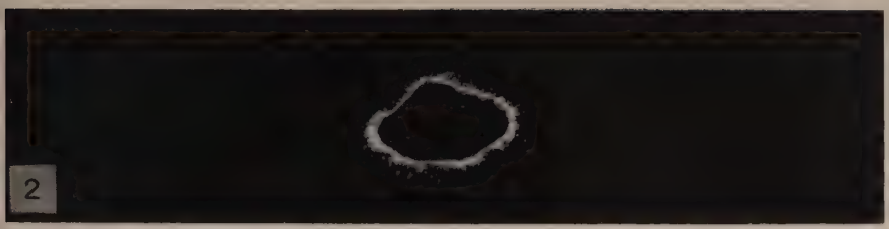
Photomicrographs of 8-day cultures of chick heart fibroblasts in Carrel flasks.

2 Maximum proliferation in media dialyzed against Ringer-glucose solution without subsequent pH adjustment. × 7.5.

3 Restoration of outgrowth in Ringer-dialyzed media by elevation to pH 7.4 with added Na_2CO_3. × 7.5.

4 Comparable outgrowth in undialyzed media. ×6.

All photomicrographs were taken by Mr. Victor Duran.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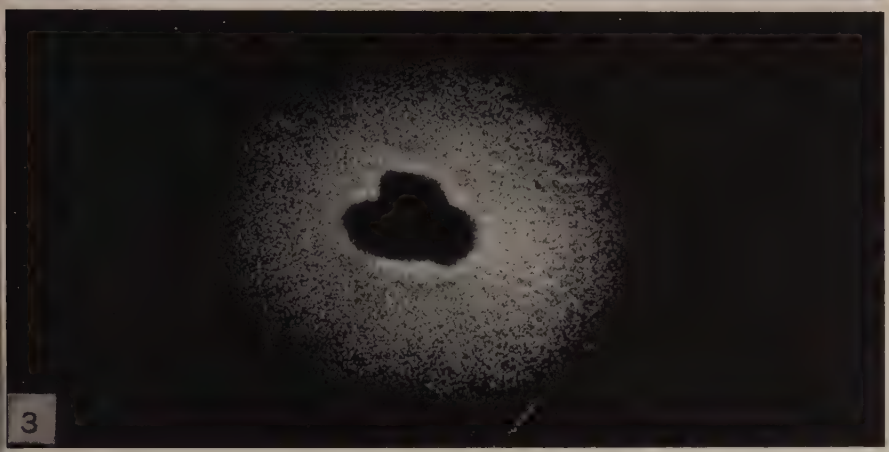
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4

EXCITABILITY RELATED TO SPIKE SIZE IN CRAB NERVE FIBERS[1]

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FOUR FIGURES

Apart from the giant fiber synapses of some invertebrates, synaptic regions typically have very small unmyelinated fibers on one or both sides of the synapses. Some authors have found it necessary to postulate that certain synaptic phenomena may be interpreted partly in terms of probable properties of these fibers (Lloyd, '50; Lorente de Nó, '50; Easton, '47). In order usefully to pursue such hypotheses, one would wish to know in what way the smaller fibers are physiologically different from the larger fibers.

The final demonstration by Huxley and Stämpfli ('49) that vertebrate myelinated fibers are active only at the nodes constitutes a difference that may be shown to have great significance with regard to the fate of the action potential in the nerve terminals that do not have nodal excitation. Among the invertebrates, however, non-nodal propagation is the rule, and in this case, it is simplest to assume that the small terminals of a fiber are similar to the parent fibers. If this be true, then the comparison of unmyelinated peripheral fibers of the same kind but of different sizes may reveal some differences that may be applied to the synaptic problem.

The account that follows describes a relation between the optimal stimulus duration and the observed spike size (the latter is assumed to be proportional to the fiber diameter).

[1] Acknowledgment is made of funds provided for support of this research by Initiative 171, State of Washington, U.S.A.

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PROCEDURE

Small bundles of up to 50, but usually less than 10, nerve fibers were prepared from the leg nerves of *Cancer magister*. The crabs were obtained from local fish handlers in the Seattle area, and kept in tanks of sea water at about 12°C. for several weeks. Only nerves from active animals were used.

To isolate the nerves, the muscle attachments and the joints were cut and the successive segments of the leg were detached. Usually some of the fibers left on the propodite (penultimate segment) were teased apart in sea water and a bundle was selected for its small size. At this level the nerve consists of not more than 5 efferent fibers to the flexor and extensor muscles of the dactyl, as well as a large number of afferent fibers of various sizes. Fiber bundles were also obtained from those left on the dactyl (final distal segment). These fibers are all afferent.

For stimulation and recording, the nerve bundle was mounted on the 0.3–0.5 mm edges of hardwood sticks that had been saturated with sea water and fixed into the ends of glass tubes filled with sea water. Into the other ends of the tubes were placed relatively non-polarizable Ag-AgCl electrodes that were freshly prepared before each experiment. From sea water the nerves were lifted by means of a rack and pinion device into a supernatant layer of oil which served as a dielectric insulator to permit observation of the action potentials. The experiments were carried out at 14–15°C.

Square-pulse stimuli in steps from 0.02–50 msec. (see table 1) were delivered from a Grass C_3 Stimulator. In order to assure sufficient power from the stimulator to excite the nerves at the shortest pulse durations, and to avoid distortion of the longer pulses, the stimulator output was used direct; neither shunting resistors to provide constant current, nor transformer for isolation of stimulus was used. To calibrate the output, the square pulse was delivered directly to the C.R.O. from the stimulator, and the height of this pulse was

measured at successive readings of the stimulator intensity control. These heights were used in constructing the strength-duration curves.

Action potentials were observed on the screen of a Dumont 208 C.R.O. after passing through a Grass P_3 preamplifier. They were also monitored from a loud speaker. Some difficulty was occasionally experienced from excessive "spontaneous activity," particularly from the small fibers. If it did not interfere with the observation of spikes produced by stimulation, the spontaneous activity was disregarded.

From the determinations of intensity, as a certain height on the C.R.O. screen, a quantity directly proportional to the energy involved in the stimuli could be calculated according to the following considerations:

The energy E involved in the transfer of a quantity of electricity dq ($=idt$) between a potential difference V (constant for the duration of the square pulse), may be expressed by: $\int dE = V \int dq = V \int idt$

If the resistance of the nerve be assumed to be ohmic, then $i = \frac{V}{R}$; and if the resistance be assumed constant during the square pulse, then $\int idt = \frac{V}{R}t$ where t = pulse duration. Hence $E = V^2t/R$.

V^2t, directly proportional to the energy involved, was calculated for each threshold stimulus, and the results were plotted as shown in the figures.

RESULTS

An attempt was made to discover the threshold voltage for individual fibers or small groups of fibers when stimuli of various durations were used. In the ideal situation, a strong stimulus of 0.02 msec. duration produced a characteristic spike that could be identified by its typical size and shape on the C.R.O. screen through subsequent successive stimulations at decreasing intensities and increasing durations up to 50 msec. At the longer durations small spikes of

low velocity began to come in at intensities subthreshold for the larger, faster spikes. Because the shorter pulses excite the larger spikes first, the small fiber activity becomes overwhelmed and confused in the declining phases of the faster spikes on which they were superimposed. Only occasionally when very few fibers were active, has it been possible clearly to distinguish separate fibers at the short durations of stimu-

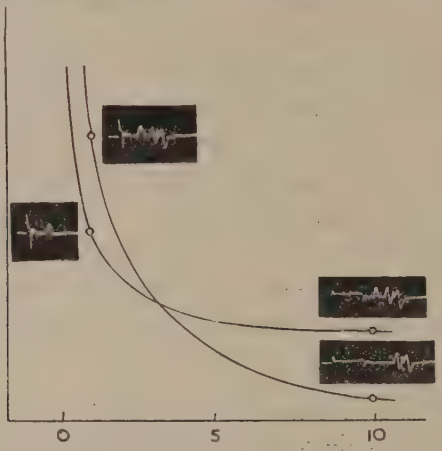

Fig. 1 Separation of two groups of fibers by strength-duration curve.
Ordinate: Intensity (a linear function of voltage required to elicit the spike).
Abscissa: Duration of shock in milliseconds.
With a short pulse, the fast fiber(s) appears at low intensity. Increasing intensity of the short pulse brings in slower fibers.
With long pulse, the threshold for the slow fiber(s) is at a low intensity. Increasing intensity of the long pulse brings in the faster fiber.

lation. Such a case is shown clearly in figure 1 which shows the type of strength-duration curve that would be expected for each fiber.

With stimuli of long duration, the separation of the spikes is much clearer. It often happens that a complex wave of constant form at short durations "falls apart" at long duration and is seen to be composed of several spikes that are "locked" at short durations, but separate as the duration of the stimulus is increased. At long durations, the smaller, slower waves appear at lower intensities; the faster, larger ones come in as the intensity is increased. As would be ex-

pected, the latencies of the various spikes vary a good deal at intensities around threshold. The variation is greatest for the smallest spikes. Since these determinations depend upon a threshold process, calculations of velocity are not therefore useful indices for comparison of the various spikes. It was clear, however, that the smaller spikes had the greater latencies.

In table 1 are given the threshold stimulus intensity-duration data for 14 different spikes, from which figures 2, 3 and 4 are constructed. The figures with their legends are self-explanatory.

When the "energy" of each threshold stimulus is plotted against its duration, the curve for each fiber has a characteristic minimum as shown in figure 2. The energy minima for the "slow responding" spikes are smaller than those for the faster spikes. The curves show clearly the manner in which fibers of different excitability may be distinctly separated by this method. The "4" curves, with minima at about 5 and 20 msec. respectively, are especially clear. Note the relative scale of energy. When a fiber of "fast response time" such as motor fiber is used (inset fig. 2) the stimulus energy is very high, and the minimum energy threshold excitation time very small. Inset figure 2 also illustrates the effect of interelectrode distance upon the minimum. At small distance, the curve becomes flat and the minimum difficult to establish.

For each fiber, the point of minimum stimulus energy was located on the energy-duration curve (see samples in fig. 2). These points are plotted in figure 3. Each point represents a different fiber. There is a good deal of scatter that may be attributed to the differences in interelectrode resistance, which was not determined. An attempt was made, however, to use fiber bundles of similar size, and to use a constant interelectrode distance. There is a tendency for those fibers preferentially excited by long durations to require less energy for excitation than is the case in fibers responding more easily to stimuli of short durations. The differences among

TABLE 1

"V"

t	1	2	3		4		5		6		7	8	9
0.02	302	380	182		200		350		820		390	460	420	670
0.05	116	174	75		88		132		292		278	288	266	252
0.10	60	92	44		48		72		144		88	97	89	129
0.20	34	58	20		24		40		80		50	55	48	86
0.50	19	28.5	12.4		12.4		18		35		23	27	25	37
1.00	8.5	17.8	8.2		7		11	11	19.8		13.3	16.6	14.4	22.7
2.00	4.5	9.8	5.8	5.6	4.5		6.7	5.9	11.7	8.5	8.3	11	8.5	16.4
5.00	2.5	4.8	4.5	3.0	2.4	2.3	4.0	3.6	6.9	4.0	4.9	7	4.5	11.9
10	1.72	3.0	4.2	2.2	2.1	1.6	3.0	2.3	6.7	2.0	3.66	6.7	3.0	11.2
20	1.36	2.25	4.2	2.34	2.36	1.06	3.18	1.62	..	0.8	3.34	8.0	2.3	11.6
50	1.20	1.58	4.2	2.64	2.75	1.06	...	1.36	..	0.8	3.29	8.5	2.3	12.4
E_{min}	25	90	65	45	27	20	75	45	240	20	125	230	80	500
t_{min}	9	11	1.5	7	5	20	6	15	4.5	35	5	3.5	11	1.5
V_{min}	1.7	2.9	6.6	2.6	2.3	1	3.5	1.7	7.3	.5	5	8.1	2.7	18
Spike	200	200	1500	150	700	50	500	75	200	200	1150	450	1000	2700

Threshold strength-duration combinations of square pulse stimuli for a number of spikes.

t = durations of square pulses in milliseconds.

Numbers heading vertical columns represent separate fiber bundles, in which one or two spikes were followed as shown.

The vertical columns represent relative intensities (a direct function of the voltage) necessary for threshold stimulation at the indicated durations.

$E_{min} = (V^2t)_{min}$ relative energy required for threshold stimulation at minimum point on V^2t vs t curve.

t_{min} = the threshold square pulse duration at which V^2t is a minimum.

V_{min} = the threshold intensity at which V^2t is a minimum.

"Spike" = size of observed action potential in microvolts.

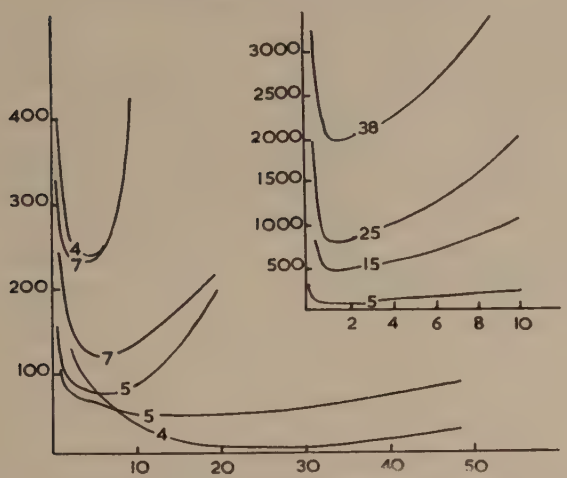

Fig. 2 Typical curves resulting from transformation of several strength-duration curves into energy-duration curves. 4, 7, 5, represent curves of different pairs of fibers in each of three different bundles of nerves. Trough of each curve represents duration of shock at which the nerve required least energy for excitation.

Inset: Effect of interelectrode distance upon the energy-duration curve in single motor fiber. Figures on curves represent interelectrode distance in millimeters.

Ordinate: Energy $(V^2t)_{min}$ of the stimulus. See text for explanation.

Abscissa: Duration of the stimulus.

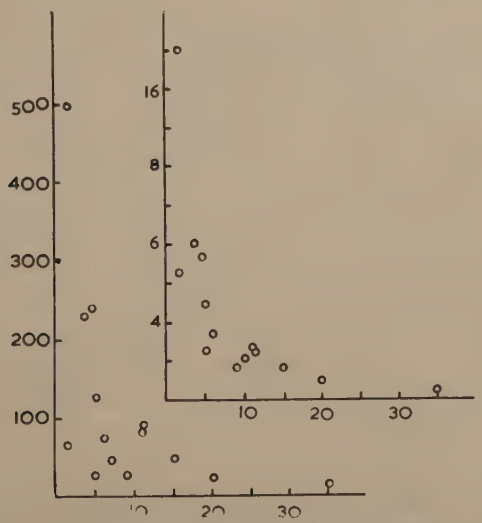

Fig. 3 Distribution of energy minima for fibers recorded in table 1.

Ordinate: Energy $(V^2t)_{min}$ of the stimulus. See text for explanation.

Abscissa: Duration of the stimulus in milliseconds.

Inset. Ordinate: The voltages at which the energy required for threshold stimulation is at a minimum. *Abscissa:* As in 3.

the fibers are more clearly shown in figure 3 (inset), which presents the same data as figure 3, except that instead of E_{min} the voltage corresponding to each E_{min} has been calculated and is plotted on the ordinate. The curve is reminiscent of the strength-duration curve of a single nerve, but it represents the combinations of strength and duration at which the energy necessary to elicit a response is at a minimum for a number of fibers.

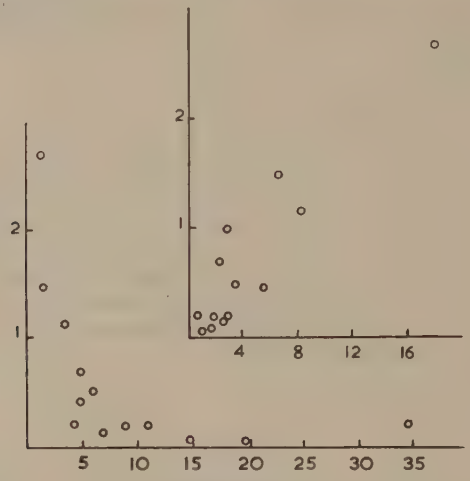

Fig. 4 *Ordinate:* Spike size in millivolts.
Abscissa: Duration of pulses providing stimulation with minimum energy for each spike.
Inset. Ordinate: As in 4. *Abscissa:* Voltage of pulses providing stimulation with minimum energy for each spike.

In figure 4 may be seen the relation between the spike size and the minimum energy threshold excitation time. It is evident that the rule: "small spike size, long characteristic duration" applies fairly well. The relation between the spike size and the intensity at minimum energy threshold is possibly linear.

DISCUSSION

The interpretation of the shape of the strength-duration curves and the relation between curves for small and large fibers may be made in terms of classical principles of core conduction (Katz, '37).

The shape of the general curve: when a nerve is stimulated at a point, a finite time is required for the electrotonus to spread and grow away from the point of stimulation. The spread is limited by the resistance and capacitance of the membrane. When the shock is very brief, there is not time, during the course of the stimulus, for the local excitation to spread sufficiently to induce a propagated process, i.e., a critical area is not yet activated. But if the brief stimulus is intense enough, it causes a profound local depolarization which, even after the stimulus ceases, maintains the current flow into the depolarized area, discharging the adjacent regions; and if the area discharged is sufficiently large, then a propagated response is produced.

If a stimulus of longer duration is used, it may excite at a lower intensity, because time is allowed for the electrotonic spread to depolarize a larger area, and so the degree of depolarization at the center (a function of the stimulus intensity) need not be as great as it must be with a stimulus of shorter duration.

The nature of strength-duration curves for fibers of different excitabilities: To explain the differences between the curves for the small and the large fibers, it is only necessary to assume that the small fibers require the application of less energy for depolarization to be accomplished and that electrotonic spread is slowed in the small diameter fibers because of the high internal resistance. The first assumption is supported by the fact that the recorded action potentials of small fibers are smaller than those from large fibers (Gasser, '41); i.e., the energy of the normal propagation mechanism — the impulse — is smaller (even though the actual membrane voltage may be the same), also the action potential occupies less area of nerve in the small fibers (this can be calculated from action potential durations and velocities in Gasser, '41, and in the present work). From this it is reasonable to assume that the critical area that must be depolarized to produce a propagated impulse is less in the smaller fibers. For a small fiber, then, a stimulus of long

duration is optimal because time is allowed for the slower electrotonic spread, and a low intensity is required because the energy required to depolarize the critical area of membrane is small. For a larger fiber, a shorter stimulus is optimal because electrotonic spread occurs more rapidly through the lower internal resistance.

The fraction of applied current that can flow through the membrane is one limiting factor in the production of a sufficient local response. This will be inversely proportional to the internal (core) resistance. For the larger fiber there will be more current flow because the cross-sectional area is greater. In the larger fiber a larger fraction of a given stimulus will flow where it will be effective in stimulation instead of being shunted through the adjacent tissues. This accounts for the fact that, with the stimuli of short duration, larger fibers are excited by a lower voltage than are smaller fibers.

It has not been necessary to invoke the concept of accommodation to interpret these data. It is usual to describe as "accommodation" the general unresponsiveness of nerves to slowly rising or constant stimuli of long duration. In the present case, the larger fibers may be said to have a more effective accommodation mechanism than the smaller ones have.

Accommodation may be considered a repolarizing process that is faster and more effective in larger nerve fibers. This may be interpreted in terms of the surface to volume ratio. This ratio operates to the disadvantage of the smaller fibers; i.e., if we assume that the energy available for the work of repolarization is proportional to the volume of the fiber, then the available energy per unit surface area is less for the smaller fibers than for the larger ones. The efficiency of the polarizing mechanism in different fibers may in fact be proportional to the fiber diameter, and a fiber 100 μ in diameter, will then be 100 $\times$ as efficient in maintaining its surface polarization as a fiber 1 μ in diameter, other things being equal. In well-protected, myelinated fibers, where the active

areas are restricted to bands 1 μ (or less) wide, and spaced at 1 mm intervals (Huxley and Stämpfli, '49) the strain for maintenance of polarization is reduced to insignificant levels, and such fibers have notoriously good accommodation by comparison with unmyelinated fibers of invertebrates (Solandt, '36).

It is of interest to consider the differences in the fraction of the total applied current that flows through the different fibers. External shunting is greater for the small than for the large fibers, and so a smaller fraction of the applied stimulus is available for stimulation of the smaller fibers. Since this is so, we may conclude that the small fibers, already excitable by weaker stimuli than the large fibers, would, if the shunting effects could be corrected for, respond to even weaker stimuli. The differences between the fibers of different excitabilities must therefore be even greater than the curves reveal.

The existence of an energy minimum in the strength-duration curve for stimulation of nerve was originally pointed out by Hoorweg (1892). Lapicque ('32) at first considered using the duration at this minimum as his "chronaxie," but rejected it on the basis of his work with the crab-nerve-muscle preparation. His failure to find a minimum in this preparation may be attributed to the fact that a process too far removed from the stimulus has been used as the index of effectiveness of the stimulus. In the crab-leg-nerve-muscle-dactyl complex, the muscle requires repetitive stimulation of the nerve. Indeed, repetitive response of the nerve often occurs with strong single shocks. To this complication must be added the presence of inhibitory fibers that have a higher threshold (with short pulses at least) than the excitatory fibers. In the present experiments, by observation of the spikes, an energy minimum has been found in every case, even when the trough of the curve is rather wide, as in the case of very small fibers.

The minimum energy threshold excitation time may be considered a more suitable index of excitability than the chronaxie. Among the criticisms leveled at the chronaxie

concept has been the observation that it varies with the electrode size and the interelectrode distance. Rushton ('34) has explained the latter fact in terms of the interelectrode resistance. It seems likely that both these facts may also be interpreted in terms of the critical area (or critical number of active nodes in myelinated fibers) that must be activated, and in terms of interference with the growth of the local response in the case of small interelectrode distances. But the principal unsatisfactoriness of the chronaxie determinations is not that particular electrode conditions are required. The difficulty is first that the index of response is too far removed from the original excitation process, and secondly, the fact that the chronaxie is an arbitrary point of a curve assumed to be standard for all tissues, which is not the case (Rushton, '35).

It is of interest to consider some of the implications of the ready responsiveness of small fibers to slow, small energy stimuli. In receptors, impulses arise from a slowly developing negativity imposed by the receptor mechanism (Granit, '47; Katz, '50). Appropriately enough, the fibers thus activated are very small, unmyelinated, with little ability to resist environmental changes. In the central nervous system, and in effectors, as an impulse invades the terminals of a branching fiber, its energy will diminish as the fiber diameter decreases. A decrease in safety factor from this cause is perhaps opposed by the greater responsiveness of the small fiber to smaller, slower stimuli. To what extent this compensation may occur cannot be judged from the present data.

SUMMARY

Using square pulse stimulation, strength-duration combinations for threshold excitation of fibers and small groups of fibers in small bundles of crab nerve *(Cancer magister)* have been determined. Separation of fibers of different excitabilities has been made on the basis of the duration of square pulse stimulation that excites with minimum energy utilization. This index is suggested as more natural than

chronaxie as a measure of excitability. A plot of the intensity-duration combinations at which a number of fibers are excited with minimum energy is hyperbolic in shape. Fibers that produce small spikes characteristically are excited with small energy but require long durations of stimulus (5–50 msec.) for minimum utilization of energy. Fibers that produce large spikes require more energy, but minimum energy utilization is at short duration of stimulus (1–5 msec.).

The shape of the strength-duration curve and the differences between the curves for different fibers are interpreted in terms of core conductor theory. To account for the ease of stimulation of small fibers by long pulses, accommodation is invoked as an alternative explanation, based upon the relative efficiencies of the membrane polarizing metabolism in fibers of different diameters.

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THE HISTOPHYSIOLOGY OF THE TELEOSTEAN PHYSOCLISTOUS SWIMBLADDER[1]

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SEVEN FIGURES

INTRODUCTION

The literature on the functioning of the teleostean physoclistous swimbladder reveals a controversy as to whether or not one or more gases are secreted into the bladder cavity against diffusion gradients by means of a physiological energy mechanism. Of the comparatively recent workers, Ledebur ('28, '29, '36, and '37) is inclined to believe that active secretion does occur. Jacobs ('30, '32, '34, and '35), on the contrary, believes that the "secretion" of the gases can be accounted for on the basis of ordinary laws of gas dissociation and diffusion from the blood. Both agree in their concluding review articles that the exact method of gas release is undetermined. Since their time, contributions have been made by Saupe ('40), Black ('40, '42, and '48) and Fange ('50), again without a definitive theory of gas release being developed.

By intensive study of one form of fish with a variety of techniques, it was hoped that a more conclusive theory could be developed. *Fundulus heteroclitus* was chosen because previous work (Copeland, '48 and '50) proves it to be a hardy fish easily studied under laboratory conditions. It has a physoclistous bladder that is quite active physiologically,

[1] Preliminary report submitted at the Cleveland Federation Meetings (Copeland, '51).

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Black ('48) having shown that the difference in specific gravity between sea water and fresh water is sufficient to stimulate the swimbladder to a compensatory release or absorption of gases.

MATERIALS AND METHODS

Fish were obtained from a local baitshop under the synonym "mummychug" and acclimatized in a 50-gallon tank of aerated sea water in a greenhouse. For experimental work, healthy female fish of medium length (ca. 9 cm) were removed from the stock tank and adapted in smaller aquaria (15-gallon) filled to a depth of 6 inches with aerated one-third strength sea water. It was felt that a depth of 6 inches would have minimal pressure effects on the bladder. Food was given at three-day intervals in the form of chopped *Venus mercenaria*.

To tap a swimbladder for removal or addition of gases, the fish was held down on a plate of glass over a strong, focussed light which clearly outlined the bladder. A number 27 hypodermic needle attached to a 1 ml tuberculin syringe could then be inserted at an angle under the scales and into the posterior part of the bladder, avoiding the secretory epithelium and associated vascular supply. Barometric equilibration was obtained by rotating the plunger and in the case of maximal deflations, the abdomen of the fish was pressed to prevent suction. In complete deflations the bevel of the needle was oriented facing toward and close to the dorsal wall of the swimbladder. As the bladder was emptied and the ventral wall collapsed, the chances of occluding the needle tip before completing the deflation were minimized. The head was wrapped in damp cotton to protect the gills. Fish so wrapped offered little or no resistance to the needle.

Gas analyses were made with the micro-method of Roughton-Scholander ('43). Difficulty was experienced in obtaining accurate carbon dioxide determinations in the lower ranges. To minimize this factor, all analyses were done by a rigid routine with careful attention to temperature control. Thus,

relative accuracy of carbon dioxide readings were obtained, but not absolute accuracy.

Various standard cytological techniques were used. They are noted at appropriate places in the following results. The only technique to give results pertinent to the interpretation of experimental results in this paper was the MacManus ('48) periodic acid-Schiff reaction for glycogen. Fixation was made in cold, agitated P.A.F. (picric acid saturated in absolute alcohol, to 10 parts of which one part of formalin was added just before used). Control slides were digested in diastase buffered to neutrality.

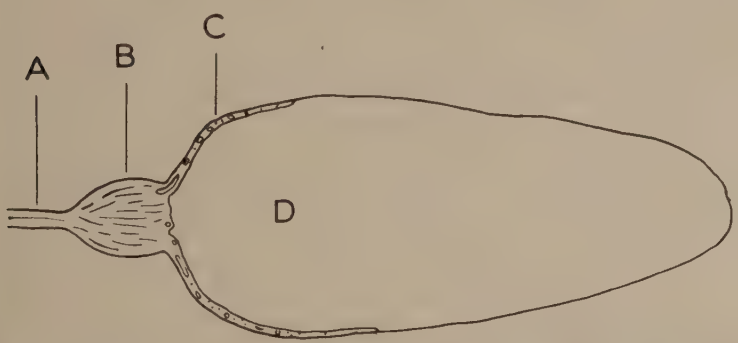

Fig. 1 A diagram of a sagittal section of the *Fundulus heteroclitus* swimbladder. Anterior is to the reader's left. A, afferent and efferent vessels. B, double rete. C, secretory epithelium. D, lumen of bladder.

RESULTS

Morphology of the normal swimbladder. Fundulus is rather exceptional in that the rete and the secretory epithelium are almost isolated from each other (fig. 1). In most fish the rete closely overlays the epithelium so that the two are difficult or impossible to separate. Of the wide range of fish described by Woodland ('11) *Fundulus* most closely resembles *Syngnathus acus*. The rete is at the anterior end and the epithelium spreads from it to cup the anterior end of the bladder. There is no vascular supply intimate to the lumen of the bladder other than that in the secretory epithelium. There is no oval window and associated capillary bed in *Fundulus*.

Thus, the question of the possible role of the oval window in the regulation of volume (Evans, '25; Meesters and Nagel, '34; and Saupe, '40) is not a complicating factor.

The double rete (red gland) structure is composed of endothelial tubules with a small amount of connective tissue and a sprinkling of granule containing cells that are assumed to be a part of the large population of leucocytes to be found in fish tissues. No significant changes were noted in the cells of the red gland.

The secretory epithelium is composed of large cuboidal type cells that show a discouraging vacuity after the usual cytological techniques. Fixation by Regaud's method, followed by postchromation and by staining with Altmann's acid fuchsin does not reveal recognizable mitochondria. Preparations according to Ludford do not show Golgi structures. Fixations in Zenker's fluid or in basic lead acetate followed by dilute toluidine blue show no metachromasia. Alkaline phosphatase preparations show no localization of that enzyme in the cytoplasm of the cell. Of interest, though, is the occasional demonstration by the phosphatase technique of intercellular tubules that look much like bile canaliculi. Acid phosphatase tests proved negative, but this is not conclusive since the complete range of substrate and pH variation was not checked. Routine haematoxylin-eosin preparations reveal a faintly acidophilic, flocculent, non-granular cytoplasm.

Composition of gases in undisturbed fish. It is very difficult to determine what could be considered the "normal" or usual gas ratio in undisturbed fish. Once stimulated to secretion of gases, the bladder probably requires several weeks to a month for complete equilibration (fig. 2). Fish kept in the laboratory and adapted in shallow tanks for a week or more show the following ranges: carbon dioxide, 0–5%; oxygen, 12–23%; and nitrogen, 72–88%. The most valid determinations are from 4 fish kept in a greenhouse tank of sea water for three months. At the end of that period they were healthy and alert. The bladder gases were respectively: carbon diox-

ide, 2.7, 1.3, 1.0, 5.3% (av. 2.6); oxygen, 20, 22, 23, 21% (av. 21.7); and nitrogen, 77, 76, 75, 73% (av. 75.2).

Gases released into the active bladder. To check the relative percentage of released gases, the bladder mechanism

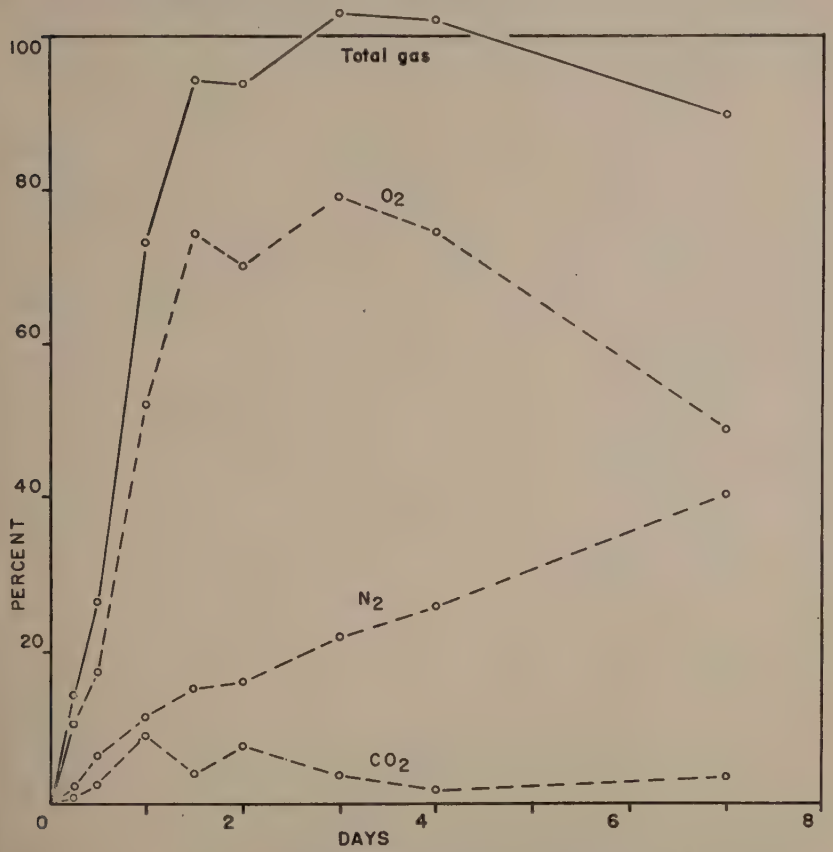

Fig. 2 Recovery from a single maximal deflation. Each point is the average of three fish. The individual gases are plotted as the relative amounts of gas to be found under each percentage of total gas recovery. (Per cent total recovery × per cent of each gas present.)

was stimulated by complete deflation of the organ. Thus, at least initially, there was no doubt that all of the gases present in the refilling swimbladder were de novo and related to the specific activity of the secretory mechanism.

Two approaches were used. One was to trace the normal recovery from a single, maximal deflation. For this, gases were removed at zero time from a number of fish, measured and analyzed; then at intervals thereafter successive fish were checked to see the progress of regeneration, both as to volume and percentage of gases. The results are plotted in figure 2.

TABLE 1

Gas data for three groups of fish repeatedly deflated by three different time schedules

12 HOUR INTERVALS				24 HOUR INTERVALS				48 HOUR INTERVALS			
I	II	III	IV	I	II	III	IV	I	II	III	IV
100.0	84.2	.95	14.7	100.0	85.8	1.46	12.9	100.0	86.4	1.2	12.5
28.8	17.5	14.5	68.0								
16.2	16.9	11.9	71.3	61.0	22.2	17.5	60.1				
23.7	16.3	13.5	70.4								
21.8	15.0	13.5	71.5	41.0	15.9	18.2	65.9	107.0	21.4	13.9	64.7
24.8	16.2	13.0	70.6								
15.9	14.3	12.9	72.8	48.5	12.7	18.0	69.2				
20.0	15.8	11.6	73.0								
22.4	17.8	11.6	70.7	55.0	11.9	18.2	70.0	103.0	13.0	15.9	71.2
20.0	14.6	11.2	74.4								
14.4	15.7	11.3	73.0	41.0	13.5	15.7	70.8				
12.5	17.8	10.6	71.7								
21.0	17.8	10.0	72.3	40.0	14.7	16.3	68.8	102.0	15.3	13.7	71.0
21.0	16.3	8.9	75.0								
17.7	17.3	9.7	73.4	29.2	15.4	13.5	71.0				
14.4	24.8	7.5	67.8								
10.0	24.8	4.6	70.5	18.7	17.9	11.3	71.0	75.0	15.3	12.4	72.5

I = per cent total recovery of gases. II = per cent nitrogen. III = per cent carbon dioxide. IV = per cent oxygen. Each value is the average of three and, in some cases, four fish. The first figure in each column is the initial or control value.

The other approach was to put the secretory mechanism under prolonged stimulation by removing all the gas at repeated intervals, keeping the gland continuously active. Three series were tested, using 12-hour intervals in one, 24 hours in another, and 48 hours in still another. The results are reported in table 1. For the purpose of discussion, the data of the intermediate series (24-hour) is also graphed (fig. 3).

The purpose of the latter series of experiments (repeated deflations) was to see if the secretory mechanism could be exhausted by continuous stimulation. A definite downward "break" occurred at 6 days for the 24 and 48 hours repeated deflations and at 6.5 days for the 12-hour stimulations. Such close agreement in three concurrent series suggested the

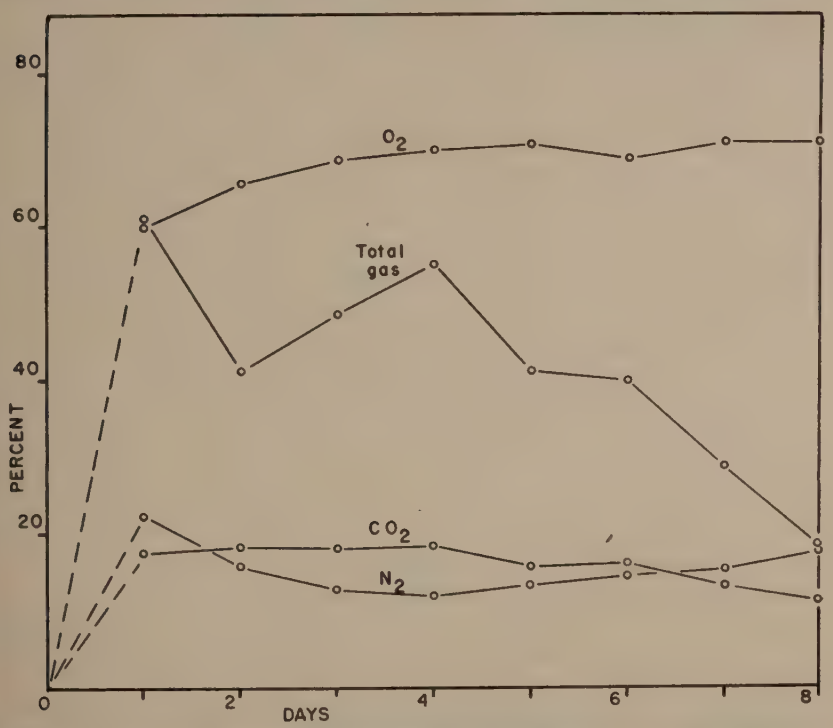

Fig. 3 Recovery from repeated maximal deflations based on the 24-hour interval data in table 1. Total recovery and individual gas recovery plotted directly as percentages.

possibility of a common factor such as feeding. To check that possibility another series of 8 fish were deflated at 24-hour intervals. Four of the fish were fed, 4 were not. Both groups showed a break or beginning of definite exhaustion at 7 days. Food apparently is not a direct factor.

From other observations the conclusion is reached that to obtain the phenomenon of exhaustion it is essential to

repeatedly deflate the swimbladder at no longer intervals than 48 hours. Random deflations that allow occasional intervals of recovery longer than 48 hours do not always cause exhaustion of the secretory mechanism.

One report in the literature that appears to be at variance with the results of my own and other investigators of gas regeneration is that of Meesters and Nagel ('34) who found that the European river perch, *Perca fluviatilis,* produces as high as 80% (72–86%) carbon dioxide. To repeat their work, yellow perch, *Perca flavescens,* was used. The two species are very closely related and are considered by some authorities to be subspecies (personal communication from Loren P. Woods, Curator of Fishes, Chicago Natural History Museum). Six perch were kept in a healthy condition in running tap water for two months, then were completely deflated. The average composition of the removed gases were: carbon dioxide, 0.2%; oxygen, 19.4%. At 24 hours there was an average recovery of 45% (range 36 to 55) of the original volume. The gas percentages were: carbon dioxide, 3.1 to 7.0 (av. 5.2) and oxygen, 37 to 58 (av. 45). The results are like those found in *Fundulus* except that recovery is slower and the oxygen and carbon dioxide concentrations lower. Certainly, they do not agree with the results reported by Meesters and Nagel.

Gases related to the absorption mechanism. To test the role of gases in the act of physiological deflation, bladders were overinflated. To maintain prolonged inflation and at the same time employ physiologically "normal" gases, the following stratagem was employed. Starting with 12 fish, the gas was removed from three. A minimal amount from each of the three was analyzed and the residual used to overinflate the remaining 9 fish, by injecting 0.15 ml into each. That amount represented about one-quarter of the average volume of the bladder in fish used throughout this work. On the next day, air from two fish (one analyzed) was injected in the remaining 7, maintaining their overinflated state. On the second day, two (both analyzed) were used to overinflate the remaining

5. On the third day, one (analyzed) was used to overinflate 4. The 4th day, one (analyzed) into three. The 5th day, the remaining fish (three) were analyzed. The changes in gas percentages associated with continuous stimulation of the gas absorption mechanism are shown in figure 4.

Influence of individual gases on the secretory mechanism. Particularly to test the possible oxygen dissociative role of the high level of carbon dioxide in the lumen of the bladder, the following was done. The gas was removed from 9 fish (three groups of three each) and measured with the needle

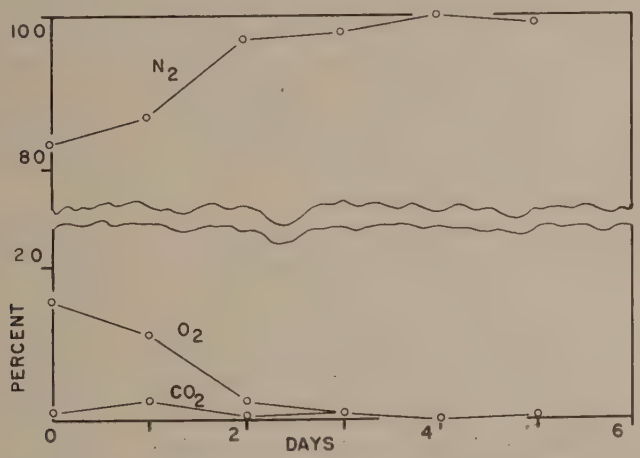

Fig. 4 Response to continual "normal" overinflation (see text). Control determinations = average of 3 fish; 1 day = 1 fish; 2 day = 2; 3 day = 1; 4 day = 1; 5 day = 3.

still inserted into the bladder. In the first group, one-quarter of their own gases was returned. In the other two the syringe barrel was disconnected and another barrel substituted to return one-quarter of the initial volume as pure oxygen in the second group and pure carbon dioxide in the third group. Thirty hours later the percentage recovery of volume under the influence of the various gases were: own gases, 104, 94, 103 (av. 99); oxygen, 95, 99, 100 (av. 98); and carbon dioxide, 71, 102, 53 (av. 70). The percentage of recovery includes, of course, the inserted gas (i.e., 25% volume present at zero

time). The experiment was repeated at a later date with 5 fish in each of the three groups. The lower temperature prevailing at that time is reflected in smaller degrees of recovery. The recovery percentages at the end of 29 hours were: own air, 78, 73, 65, 90, 27 (av. 64); oxygen, 75, 61, 81, 60, 58 (av. 67.5); and carbon dioxide, 25, 44, 72, 68, 52 (av. 52). There is considerable overlap, but these observations may have some significance.

The release of gases against pressure. It was pointed out by Scholander (conversation at Woods Hole, 1950) that at approximately 4 atmospheres' pressure, even if all the oxyhaemoglobin is dissociated, there would be no gas release (i.e., bubble formation). All of the dissociated oxygen would remain in plasma solution at those pressures. From this viewpoint he was very much interested in the swimbladder gas compositions to be found in fish caught at considerable depths (Scholander, '51).

A. C. Redfield confirmed Scholander's calculations and kindly offered me the use of the pressure bomb in the Woods Hole Oceanographic Institute. Two fish put at a total of three atmospheres' (i.e., ambient plus two atmospheres) pressure for 24 hours showed a loss of about one-half of their bladder volume. To check the relative roles of secretion vs. loss, the experiment was modified as follows. Two fish were completely deflated and put at a total of 5 atmospheres' pressure for 24 hours. Any gas found in the bladder under those circumstances would have been secreted. No gas was found, so it can be concluded that, at least in the case of the two fish, the bladder could not inflate against 5 atmospheres' pressure.

Intracellular glycogen as related to the activity of the secretory epithelium. In looking for possible energy mechanisms in the swimbladder, glycogen tests were tried with spectacular results. A normal bladder epithelium, considered to be slightly active (see Discussion), showed a localized deposition of the carbohydrate (fig. 5). There is a polarization in that there is usually a clear, glycogen free layer in that

part of the cell adjacent to the vascular supply. This, no doubt, corresponds to the striated layer described by Woodland ('11), though no striations were observed by the techniques reported here. A bladder stimulated to extreme gas secretion by complete deflation showed an exhaustion of glycogen (fig. 6). Those stimulated to absorption of gases showed a storage of the material (fig. 7). The diastase control slides were consistently negative.

DISCUSSION

Of the gases released into the swimbladder, it is apparent that oxygen and carbon dioxide are linked to a physiological mechanism, whereas nitrogen is not. Study of figure 2 shows that the original volume is reestablished by an active input of oxygen and carbon dioxide, with nitrogen lagging behind. Nitrogen recovers along a straight line that is characteristic of a passive diffusion gradient. The passive diffusion is probably enhanced in the first few hours by the pull of the displaced organs and abdominal wall against the collapsed swimbladder. Once the original volume has been established, there is a gradual loss of the first two gases as nitrogen continues passively to enter. Return to normal gas ratios requires approximately two weeks.

The results of my work in correlation with the literature supports the theory that the high level of oxygen and rise in carbon dioxide can be accounted for by dissociation of oxyhaemoglobin and carbonates under the influence of a pH shift in the acid direction. The double, parallel rete in the red gland serves as an interchange device that can be very efficient because of the two principles involved (slow flow peculiar to rete and contra-flow in the two sections). There, the afferent blood is preconditioned by the efferent blood, released carbon dioxide and oxygen crossing over with the "acidifying" component produced by the epithelium. The cyclic system so established can raise the total pressure of carbon dioxide and oxygen in the epithelium and at the same time have some protective value in that the efferent blood

will have a lowered "acidity" value when it reaches the systemic circulation.

Several references in the literature support the acid-dissociation theory insofar as carbonic acid may be a participating factor. Hall ('24) has noted that an increase in ambient carbon dioxide causes an increase in bladder volume. He also says that a distilled water extraction of active swimbladder is slightly more acid than that from inactive swimbladder. Black ('42) reports that as carbon dioxide is increased in the water, fish lose the ability to utilize oxygen from the swimbladder under conditions of asphyxiation. High vascular carbon dioxide pressures (acidity) tend to keep oxygen in the swimbladder.

The disappearance of glycogen from the actively secreting epithelium can be interpreted to support either of the opposing theories mentioned in the introduction. It may be used as a substrate for an energy mechanism to mobilize the oxygen, or it may be oxidized to an organic acid for a more conventional dissociative mechanism. I am inclined to the latter view and the indications are that an organic acid of a more intermediary nature than carbonic acid is the agent. Warburg determinations made by Eric Ball on *Scup* swimbladder using glucose as a substrate indicate that the carbohydrate is not completely broken down to carbon dioxide (personal communication).

Ball's finding gives a clue to the apparent discrepancy between Black's ('42) statement that carbon dioxide increase in the water (and blood) promotes oxygen retention in the swimbladder and my own observation that pure carbon dioxide (compared to pure oxygen) within the bladder retards gas release into the inflating organ. It is apparent that the oxidative mechanism which produces an intermediate organic acid (and carbonic acid as a byproduct) is much more effective as a dissociative agent than is a high concentration of carbon dioxide alone.

In light of the preceding paragraph a very interesting correlation can be seen in figure 3. If it is assumed that the total

pressure in the swimbladder remains relatively constant (or follows the same sequence of changes during each recovery interval), then the percentage of a gas can be interpreted as the partial pressure of that gas. The partial pressure is of course quite significant in relation to secretion, absorption, or any other reaction involving the gas. On that premise, carbon dioxide is the only gas of the three that shows a correlation to total recovery in the swimbladder. Since the inflation of the swimbladder is an active process (in the sense that it can inflate to a pressure exceeding the ambient pressure), then the per cent of carbon dioxide is in direct correlation with that activity. Applying the reasoning in the preceding paragraph, the amount of carbon dioxide in the bladder indicates the degree of glycogen oxidation in the epithelium to produce the intermediate organic acid that results in a certain level of dissociation in the blood.

When the swimbladder is forced to resorb gases (fig. 4), the three gases do not leave by a common diffusion gradient. Oxygen and carbon dioxide disappear quickly and, in some cases, completely, suggesting a mechanism that preferentially removes the two gases. Again, the phenomenon can be accounted for by a pH shift, this time in the alkaline direction. The storage of glycogen (fig. 7) indicates that the acid producing system is inactivated and that in itself might cause a sufficient change in balance to produce a retardation of dissociation. Whether or not there is a mechanism to manufacture alkaline products in aid of oxygen and carbon dioxide pick-up by the blood (fig. 4) is not clear at this time.

True deep sea fish do not possess functional swimbladders. However, fish caught at considerable depths (many times greater than the critical 4 atmospheres calculated by Scholander) have been shown to have well-filled swimbladders. The hidden factor in those cases may be the one of vertical diurnal migration common to such fish. If it could be proven that the fish were at depths for at least several days before capture, then the energy mechanism theory would receive fresh support.

Scholander's ('51) observation of a relative elevation of nitrogen partial pressure in fish caught at great depths may be accounted for by a quick response of the reflexes. Fish pulled to the surface would attempt to deflate their bladders by absorption of carbon dioxide and oxygen. If the fish were not tested within a minute or two of being hooked, perceptible changes might occur resulting in an apparent elevation of nitrogen pressure.

The failure of *Fundulus* to produce gases at 5 atmospheres is indicative of a dissociative-diffusion mechanism but not conclusive. Due to time limitations, the experiment was cursory.

It is reasonable to believe that in normal, undisturbed fish the bladder has a small amount of activity to maintain a gas pressure slightly above ambient pressure. Muscular tone, activity, visceral pressure, etc., probably necessitate that. The results obtained from the 4 fish maintained in an aquarium for three months support the idea. The average per cent of oxygen (21.7) is above that to be found ambiently.

In summary, it is interesting to list the percentages, maxima and minima, observed for individual gases throughout my work on *Fundulus:* nitrogen, 100 to 8.5; oxygen, 82 to 0; carbon dioxide, 23.2 to 0. Within these ranges fall most of the data in the literature, with the notable exception of Meesters and Nagel's ('34) report of high carbon dioxide levels in *Perca fluviatilis.* As noted above, their work is not substantiated by work on the closely related American species, *Perca flavescens.*

One last factor should be mentioned as worthy of consideration and that is the matter of the invasion coefficients of the individual gases. Ege ('15) and Thorpe and Crispe ('49) have shown that on the basis of dissimilar invasion coefficients it is quite possible to have in fluid surfaced bubbles (associated with living systems) partial pressures that are at variance with those calculated for equilibria of individual pure gases. The surfaces of the bubbles shared two environments, one living, the other the surrounding water. The dual

surface is essential for manifestation of the phenomenon. It may be that the swimbladder by some physiological trick obtains the same effect. If so, then some of the odd variations such as noted by Scholander ('51) in regard to nitrogen may be accounted for. Particularly worthy of consideration would be the swimbladders possessing two diffusion surfaces, an oval window (with its capillary bed) and a secretory epithelium (with its capillary bed).

(Since submission of this paper, the following has been published: Scholander, P. F., C. L. Claff, C. T. Teng and V. Walters, 1951. Nitrogen tension in the swimbladder of marine fishes in relation to the depth. Biol. Bull., *101:* 178–193.)

SUMMARY

1. Evidence reported here, in correlation with the literature, supports the theory that the high concentrations of oxygen and carbon dioxide in secreting swimbladders can be accounted for on the basis of a pH shift to the acid side, followed by dissociation of vascular carbonates and oxyhaemoglobin.

2. There is some evidence of the reverse conditions occurring in bladders that are absorbing gases back into the circulatory system.

3. During inflation, oxygen and carbon dioxide are released by a physiological mechanism. Nitrogen enters passively in a diffusion gradient. Once the original volume is reestablished, the oxygen and carbon dioxide gradually leave as nitrogen continues to enter. Equilibration occurs in about two weeks.

4. If the swimbladder is stimulated to absorption of gases by maintaining it in an overinflated state, carbon dioxide and oxygen may be completely removed, leaving 100% nitrogen in the lumen.

5. Glycogen is normally found in the cells of the secretory epithelium. It disappears when inflation occurs and is stored when absorption of gases occurs. It is interpreted that the

glycogen is oxidatively broken down to produce the acidic pH shift associated with inflation. Its storage indicates ascendancy of the relatively alkaline (absorptive) phase.

6. In the act of inflation the per cent of carbon dioxide shows a direct relationship to the volume of recovery, this indicating a causal relationship with the inflating mechanism. It is interpreted that carbon dioxide level in the bladder indicates the extent of the oxidative break-down of the glycogen to form an organic acid.

7. *Fundulus* cannot produce free gas in the swimbladder against 5 atmospheres' pressure. This supports the dissociation-diffusion theory.

8. If the bladder is repeatedly deflated, a relatively abrupt failure of the ability to regenerate gases occurs on about the 7th day. Food is not a factor in this unexplained failure.

9. In light of the results obtained in *Fundulus,* practically all of the data reported in the literature can be reconciled to the dissociation-diffusion theory except for the work of Meesters and Nagel. Their work could not be duplicated in a closely related fish.

10. The occurrence of gases in swimbladders of fish caught at great depths is puzzling but may be accounted for by diurnal migration to shallower depths.

11. It is suggested that the dissimilar invasion coefficients of gases may play a role in obtaining otherwise unexplained gas partial pressures, especially in those bladders possessing two diffusion surfaces (capillary bed of oval window and capillary bed of secretory epithelium).

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PLATE 1

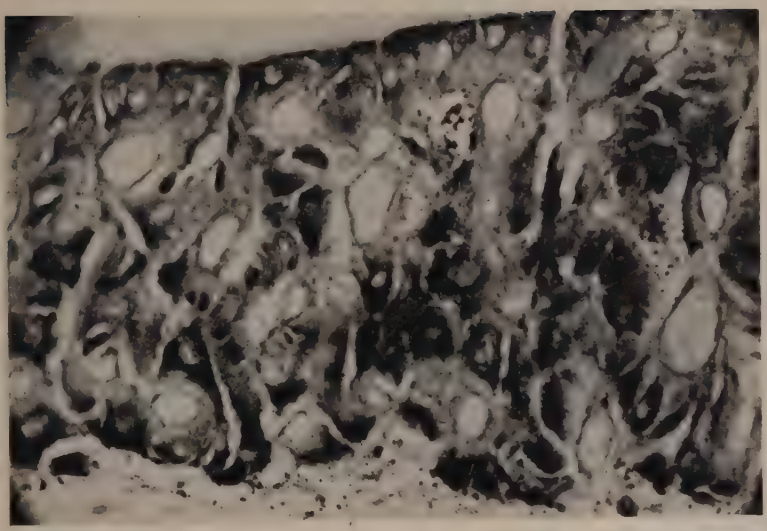
EXPLANATION OF FIGURES

5 Glycogen demonstration in epithelium of a normal fish. Note polarization in reference to the vascular supply. × 900.

6 Glycogen demonstration in epithelium of fish stimulated to secretion by deflation of the bladder. Picture is very much like that in the diastase controls, showing complete exhaustion of the glycogen. × 900.

7 Glycogen demonstration in overinflated fish. Here the epithelium is presumably absorbing bladder gases and glycogen is stored. × 900.

Photomicrograph made by Kohler illumination (Copeland, '48) with a green Wratten 61 filter and Wratten M plates.



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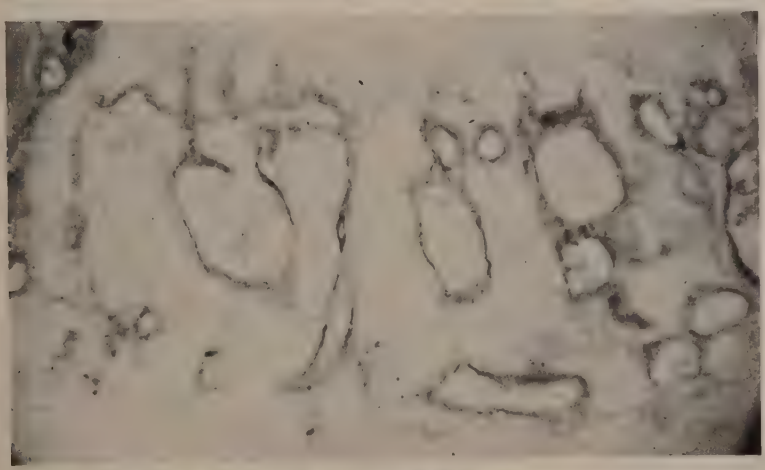

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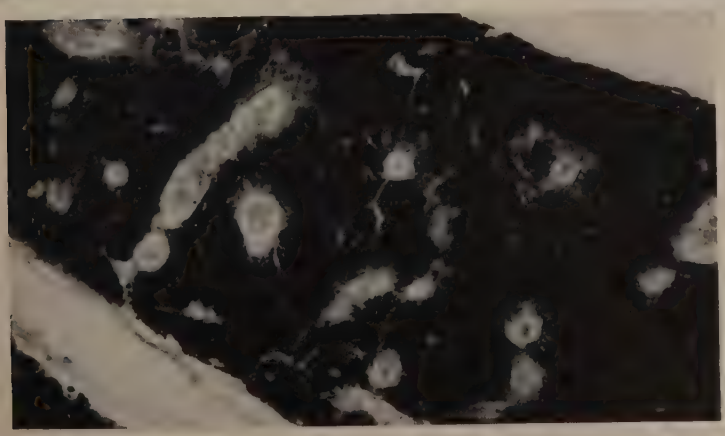

7

THE RELATION BETWEEN RIBONUCLEIC ACID AND IONIC TRANSPORT ACROSS THE CELL SURFACE

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TWO FIGURES

Exploratory studies (Lansing, Rosenthal and Kamen, '49) have indicated an association between a ribonucleoprotein complex and the cell surface. Neuberg and Roberts ('49) have proposed a mechanism for the transport of insoluble inorganic compounds from the soil into roots based upon the remarkable powers of nucleic acids and their derivatives to form complexes with other substances. It occurred to us that a process similar to that described by Neuberg and Roberts could transport ionizable materials through the cell membrane. This investigation had two primary objectives; to demonstrate by a histochemical method that a thin layer of ribonucleic acid occurs at the cell surface and to measure the influence of such surface ribonucleic acid on the uptake of ions from the extra-cellular milieu.

For the histochemical localization of ribonucleic acid the eggs of Arbacia constituted favorable material. The distribution of basophilic material, before and after exposure to ribonuclease *in vivo* was the means of localizing RNA. The uptake and retention of alkaline earth cations can be studied conveniently in the leaf cells of the fresh water plant, *Elodea canadensis*, since the vacuoles of these cells contain free oxalates. When alkaline earth cations are released from the cytoplasm by stimulation and diffuse into the vacuole, char-

[1] Lalor fellow, 1949.

acteristic insoluble oxalate crystals are formed (Mazia and Clark, '36).

METHODS

Surface RNA in Arbacia

For each experiment, two batches of fresh, unfertilized Arbacia eggs were collected in sea water. In one batch, crystalline ribonuclease (Worthington) was added to make a concentration of approximately 50 γ/ml. The solution of this enzyme had been previously heated to 70°C. for 20 minutes to eliminate proteolytic contaminants (McDonald, '48). For the second control batch, ribonuclease was inactivated by prolonged boiling, cooled, and then added in the same concentration. In all, 8 experiments were performed. At the end of 20 minutes the supernatant was decanted, the egg suspensions were fixed in absolute alcohol-formalin (9:1) for two to three hours, dehydrated, cleared, and embedded in paraffin in the usual manner. Sections cut at 6 were stained in 0.1% toluidin blue, dehydrated in tertiary butyl alcohol, cleared in xylol, and mounted in clarite.

RESULTS

The eggs of the control group were generally round but somewhat wrinkled because of the fixation in alcohol-formalin. The vitelline or outermost membrane of the egg was wrinkled and exhibited marked basophilia, staining a deep, clear blue color with toluidin blue. Subjacent to the vitelline membrane the hyaline layer showed little concentration of the dye. Underneath the hyaline layer, the cell cortex was generally characterized by a thin but distinct basophilic band not more than 1 μ thick which was easily distinguishable from the more moderate, diffuse basophilia which characterized the rest of the cell cytoplasm (fig. 1).

The sections of Arbacia eggs which had been exposed to ribonuclease *in vivo* were strikingly different from those of the controls (fig. 2). There was marked reduction in the basophilia of the vitelline membrane and the latter appeared

strongly metachromatic. This contrasted sharply with the orthochromatic color assumed by the vitelline membrane in the control preparations. The basophilia in the cytoplasm was sharply reduced, with complete absence of the cortical band of basophilic material. The disappearance of the cortical basophilic layer upon treatment with ribonuclease indicates the existence of ribonucleic acid in the cell periphery.

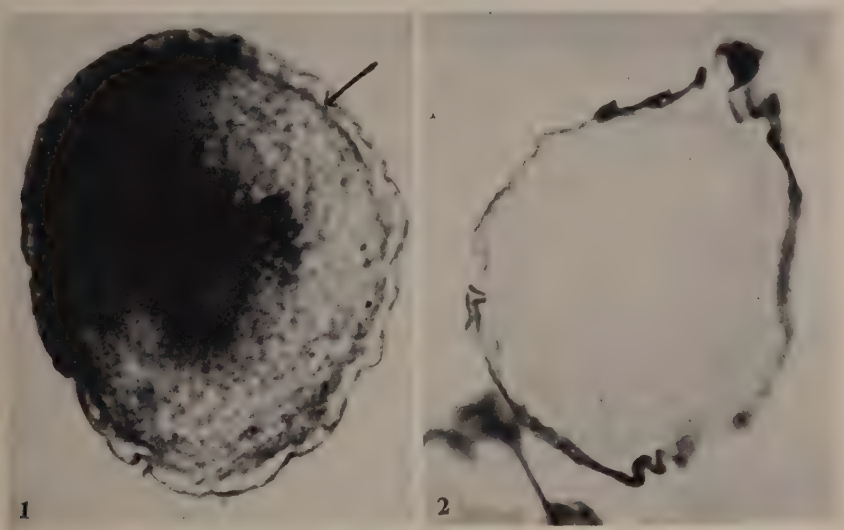

Fig. 1 Unfertilized control Arbacia egg stained with 0.1% toluidin blue. Arrow points to cortical band of RNA. × 400.

Fig. 2 Same as figure 1 but treated with ribonuclease, showing less of cortical RNA. × 400.

METHODS

Surface RNA in Elodea

As already indicated, crystals of calcium oxalate precipitate in the cell sap of *Elodea canadensis* by the combination of diffusible calcium with naturally occurring free oxalate. According to Mazia and Clark ('36) a variety of stimulating agents such as electric current, mechanical shock, ultra-violet light, heat, and osmotic changes cause a release of calcium ions from a bound state in the cytoplasmic periphery; it is

this freed calcium which participates in the formation of calcium oxalate crystals. Exposure of the Elodea cells to 0.04 molar citrate prior to stimulation blocks the formation of crystals; this is presumably accomplished by the production of a non-ionizable calcium citrate complex. However, stimulation again becomes effective in the formation of crystals if citrated cells are washed and immersed in calcium containing solutions. This is attributed to the creation of new calcium-binding in the cell periphery. The present experiment is an adaptation of these phenomena.

If ribonucleic acid is present at the cell surface and if it is instrumental in the uptake and binding of calcium ions, then the effect of ribonuclease after citrating should be to interfere with the subsequent ability of the cell surface to bind new calcium.

The following schedule was employed, with plasmolysis by sucrose and deplasmolysis in pond water as an osmotic stimulus:

EXPERIMENTAL		CONTROL
1. 0.04 M Na citrate	10 minutes	0.04 Na citrate
2. Ribonuclease, 100 γ/ml	15 minutes	Boiled RNASE 100γ/ml
3. 0.02 M Ca or Sr chloride	5 minutes	0.02 M Ca or Sr chloride
4. 0.5 M sucrose	10 minutes	0.5 M sucrose
5. Pond water.	..	Pond water

Since the quantity of precipitable calcium is a function of the position of the leaf on the stalk, that is, its age (Lansing, '42), we selected pairs of leaves from a constant position. The 10th pair from the tip of the stalk was carefully cut off with scissors and transferred to the various solutions with forceps, one member of each pair serving as the control. After deplasmolysis the leaves were mounted in pond water on slides under cover slips for microscopic examination. The leaves were systematically scanned and counts were made of all the oxalate crystals in the cells of the upper surface of the leaf. The crystals were classified as either large or small.

RESULTS

In the control Elodea leaves osmotic stimulation by plasmolysis and deplasmolysis results in the formation of characteristic intracellular calcium or strontium oxalate crystals. These crystals, which have been described in detail earlier (Mazia and Clark, '36; Mazia, '38; Lansing, '42) occur in the monoclinic or tetragonal form. The crystals of calcium oxalate were preponderantly in the form of small monoclinics and small tetragonals; the latter average $21.5 \pm 0.7\ \mu^3$. Strontium oxalate crystals generally occurred as small tetragonals and large tetragonals, with practically no monoclinics. Assuming that the large tetragonal crystals of

TABLE 1

EXP. NO.	NUMBER OF CRYSTALS				VOLUME OF CRYSTALS (μ^3)		
	Control		Exp.		Control	Exp.	Per cent reduction
	Small	*Large*	*Small*	*Large*			
1	16	16	11	6	3901	1570	60
2	14	4	8	3	1190	839	29
3	15	6	1	0	1656	22	99
4	14	4	3	4	1190	954	20
5	5	12	4	5	2775	1197	43
6	20	48	7	10	11,100	2374	79

calcium and strontium oxalate are of approximately the same size, the latter would have an average volume of $222.3 \pm 13.4\ \mu^3$ (Lansing, '42).

The exposure of the cells of Elodea to active ribonuclease invariably resulted in a marked suppression of either calcium or strontium oxalate crystal formation. Concentrations of a few tenths of a milligram per milliliter or an exposure for 30 minutes at a concentration of 100 γ/ml completely inhibited crystal formation. In order to obtain a semi-quantitative measure of the effectiveness of ribonuclease at levels just above threshold we limited the treatment to 15 minutes at 100 γ/ml. After such treatment the Elodea cells were viable as evidenced by the presence of active cyclosis. The re-

sults of a typical series are listed in table 1. In spite of considerable variability between experiments (probably due to daily and diurnal fluctuations in irritability) it is obvious that there was a significant reduction in precipitable calcium or strontium after mild exposure to ribonuclease. The effect of ribonuclease is not irreversible since treated cells produce numerous calcium or strontium oxalate crystals after standing in pond water for at least one hour.

DISCUSSION

The distribution of cytoplasmic ribonucleoprotein has been the subject of intensive investigation. According to Caspersson ('50) there is a gradient of diminishing concentration of RNA from the region of the nuclear membrane to the cell periphery (in mammalian tissue). While Caspersson's view is generally supported, several investigations tend to qualify the concept of a simple gradient. Brachet ('40, '42) in his studies on frog ova described a cortical ring of RNA which stained with toluidin blue and which was digestible with ribonuclease. Ludford's et al. ('48) photomicrographs taken by ultraviolet illumination illustrated a definite cortical zone of ultra-violet absorbing material, presumably RNA.

Analytical studies of Hogeboom, Schneider and Palade ('48) indicated that most of the RNA was associated with the particulate cytoplasmic fractions. Brachet's *in vivo* centrifugation studies showed that while much of the cytoplasmic RNA is centrifugable the cortical layer of RNA is not displaceable.

The strongest evidence for the concentration of some RNA in the region of the cell surface is derived from observations on bacterial and yeast cells. Henry and Stacey ('43) observed that the Gram-positive staining characteristic of the bacterial cell surface can be extracted by a number of agents including bile salts. Gram-positivity could be restored by immersion of the cells in the magnesium salt of ribonucleic acid. Dubos ('45) and Bartholomew and Umbreit ('44) found that treatment of Gram-positive bacteria and yeast in ribo-

nuclease resulted in loss of this surface Gram-positivity. The capsular antigen of pneumococci is inactivated by an enzyme, apparently ribonuclease (Dubos and Thompson, '38; Dubos and MacLeod, '37).

The present description of a cortical layer of RNA in Arbacia eggs is in complete agreement with Brachet's study on frog eggs, although the latter confined his enzymatic observations to fixed and sectioned material while we used ribonuclease *in vivo*. Since surface RNA has been demonstrated in cells of organisms ranging from bacteria to the highest vertebrates it seems quite possible that this is a universal feature of cell structure. The question arises as to what its function is in this location.. More specifically, is it possible that this RNA is associated with the regulation of permeability of the cell, and thus mediates intracellular synthetic processes?

Our experiments on Elodea indicate that the effect of ribonuclease is to interfere with the mechanism of uptake of calcium and strontium ions by the cell. In the light of the demonstrations that RNA exists in a number of forms as a thin cortical layer, and since Mazia and Clark ('36) and Mazia ('40) showed that the uptake of the calcium ion by Elodea depends initially upon its being bound at the surface, the conclusion is warranted that the ribonuclease acts upon surface RNA to inhibit calcium ion uptake. Although we have confined our experiments to two bivalent cations, Neuberg and Roberts ('49) have shown that nucleic acid may form complexes with organic as well as inorganic substances and have estimated that "the number of possible combinations for the ribose tetranucleotide unit turns out to be of the order of 16^{15}, this equals 10^{18}."

On the basis of a surface layer of ribonucleic acid and its immense capacity for forming complexes it is tempting to infer that the initial step in the transfer of material from the external milieu to the cell interior involves the combination of this material at the surface with RNA. This view is consistent with the hypothesis of Rothstein and Meier ('51)

that polyphosphate materials located at the surface of the yeast cell are necessary for the phosphorylative binding and transport of glucose.

SUMMARY

The two primary objectives of this investigation were to demonstrate by a histochemical method the existence of a thin layer of ribonucleic acid at the cell surface and to measure the influence of such surface ribonucleic acid on the uptake of ions from the extracellular milieu.

Staining with toluidin blue revealed the presence of a cortical band of basophilic material in Arbacia eggs which disappeared upon treatment *in vivo* with ribonuclease.

In vivo treatment of Elodea cells with ribonuclease was shown to inhibit the normal uptake of calcium and strontium ions.

The suggestion was made that the initial step in the transfer of material from the external milieu to the cell interior involves the formation of a complex between the external material and the ribonucleic acid at the cell surface.

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CONTENTS

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